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MINI-REVIEW

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Membrane targeting and translocation of bacterial hydrogenases

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Abstract Periplasmic or membrane-bound bacterial hydrogenases are generally composed of a small subunit and a large subunit. The small subunit contains a peculiar N-terminal twin-arginine signal peptide, whereas the large subunit lacks any known targeting signal for export. Genetic and biochemistry data support the assumption that the large subunit is cotranslocated with the small subunit across the cytoplasmic membrane. Indeed, the signal peptide carried by the small subunit directs both the small and the large subunits to the recently identified Mtt/Tat pathway, independently of the Sec machinery. In addition, the twin-arginine signal peptide of hydrogenase is capable of directing protein import into the thylakoidal lumen of chloroplasts via the homologous ΔpH-driven pathway, which is independent of the Sec machinery. Therefore, the translocation of hydrogenase shares characteristics with the ΔpH-driven import pathway in terms of Sec-independence and requirement for the twin-arginine signal peptide, and with protein import into peroxisomes in a "piggyback" fashion.

Key words Hydrogenase · Metalloenzyme · Folding · Enzyme complex · Signal peptide · Twin-arginine · Membrane targeting · Cotranslocation · Sec system · Mtt/Tat system

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Introduction

Hydrogenases are enzymes of widespread occurrence in bacteria (Voordouw 1992; Wu and Mandrand 1993). They catalyze the production and consumption of hydrogen gas and occupy a central place in the energy metabolism of anaerobic bacteria (Adams 1990). There are two major types of hydrogenases, namely nickel-iron (NiFe-) hydrogenases and iron (Fe-) hydrogenases. Whereas the overall structures of the two types of hydrogenase are quite different (Volbeda et al. 1995; Peters et al. 1998; Nicolet et al. 1999), some features are very similar and probably represent convergent evolution. One of these common features concerns the membrane targeting and translocation system used by the periplasmic and membrane-bound hydrogenases. These hydrogenases are generally heterodimers and are composed of a small and a large subunit. Only the small subunit has targeting signals, which contain a conserved RRxFxK motif (Voordouw 1992; Wu and Mandrand 1993). Recently, compelling evidence indicates that this twin-arginine signal peptide targets a class of enzymes, including hydrogenases, to a novel protein translocation pathway, known as the membrane targeting and translocation (Mtt) or twin-arginine translocation (Tat) pathway (Settles et al. 1997; Bogsch et al. 1998; Chanal et al. 1998; Santini et al. 1998; Sargent et al. 1998b; Weiner et al. 1998; Rodrigue et al. 1999). In this review, we attempt to summarize the salient features of the translocation of hydrogenases through the Mtt/Tat pathway in *Escherichia coli*.

Translocation of hydrogenases in folded conformation across the cytoplasmic membrane independently of the Sec machinery

Although the NiFe- and Fe-hydrogenases are unrelated at both the primary and three-dimensional structural levels, the basic architecture of their metal centers does share some similarities (Volbeda et al. 1995; Nicolet et al. 1999).

In both cases, the hydrogen activation centers are accommodated by the large subunits and are deeply embedded inside the enzymes. The iron atom at these sites is coordinated with CN and CO ligands and it is also bridged to the nickel atom in NiFe-hydrogenases. The assembly of the metal centers is a complex process and it has been extensively studied for the NiFe-hydrogenases (for review see Friedrich and Schwartz 1993; Vignais and Toussaint 1994; Maier and Böck 1996; Drapal and Böck 1998). It requires the participation of at least one specific chaperon-like protein and of a specific endopeptidase, both of which are cytoplasmically localized (Maier and Böck 1996). The large subunits possess a C-terminal extension sequence of between one and two dozen residues. The extension sequence seems to keep the precursor of the large subunit in a conformation suitable for nickel acquisition and it is removed by the specific endopeptidase following nickel incorporation (Friedrich and Schwartz 1993; Vignais and Toussaint 1994; Maier and Böck 1996; Drapal and Böck 1998). The removal of the extension sequence occurs in parallel with a conformational change of the enzyme and makes the process irreversible.

Interestingly, the depletion of the specific nickel importer, the chaperon-like protein or other components, which are coded by the *hyp* genes and participate in nickel incorporation, leads to accumulation of a hydrogenase precursor in the cytoplasm (Menon et al. 1991, 1994; Bernhard et al. 1996; Rodrigue et al. 1996a, b; Sargent et al. 1998a). Therefore, the acquisition of nickel in the cytoplasm seems to be a prerequisite for the translocation of hydrogenases. It suggests that hydrogenases are probably translocated across the cytoplasmic membrane in a folded conformation. Such a mechanism is incompatible with the Sec pathway, since this pathway can export only unfolded, single polypeptide chains (Pugsley 1993). Indeed, the translocation of hydrogenase 2 of *E. coli* is independent of SecY, which is one of the core components of the Sec pathway (Rodrigue et al. 1999).

Mechanism of hydrogenase cotranslocation

As early as 1985, sequence data revealed that the large subunit of the periplasmic Fe-hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) does not contain any known signal for export (Voordouw and Brenner 1985; Voordouw et al. 1985). It was thus proposed that the large subunit could be cotranslocated with the small subunit. Later, systematic sequence analysis confirmed the absence of signal peptide from the large subunit and revealed the ubiquitous presence, in the small subunit, of a peculiar signal peptide with a conserved RRxFxK motif. Thus, the assumption of a cotranslocation can be generalized to all periplasmic or membrane-bound NiFe- and Fe-hydrogenases (Voordouw 1992; Wu and Mandrand 1993).

In addition to the signal peptide of the small subunit, it was proposed that the C-terminal extension segment of the large subunit of the periplasmic Fe-hydrogenase from *Desulfovibrio desulfuricans* could also function as a peroxisomal targeting signal (PTS)-like targeting signal peptide and direct the translocation of this enzyme into the periplasm (Hatchikian et al. 1999; Nicolet et al. 1999). This hypothesis is based on the observation that the cytoplasmic monomeric Fe-hydrogenase does not contain this extension, which is thus not required for the metal center assembly. However, such a targeting function of the C-terminal extension seems unlikely for two reasons. First, the small subunit of the Fe-hydrogenase folds as a belt, completely embracing the large subunit, and the two subunits must be translocated as a complex (Hatchikian et al. 1999; Nicolet et al. 1999). Within the complex, the N-terminus of the small subunit is located adjacent to the C-terminus of the large subunit; thus, the two closely located targeting signals that are destined for different translocation pathways would create confusion for the cellular targeting machinery. Second, since the iron center and the nickel-iron center show a common basic architecture, some steps of the assembly of the two metal centers may be similar. Thus, as happens in the case of NiFe-hydrogenase, the C-terminus of the large subunit of the periplasmic dimeric Fe-hydrogenase may play a role in coordinating the formation of the metal center and the assembly and cotranslocation of the enzyme complex. Such a function would not be required by the monomeric cytoplasmic Fe-hydrogenase.

To elucidate the hitchhiker mechanism of the cotranslocation of hydrogenases, two models can be proposed. According to the first model, membrane targeting of the two subunits of hydrogenases 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 allows the processed large subunit to specifically interact with the membrane-bound small subunit. This interaction would trigger membrane insertion of the large subunit and formation of the complex, which then crosses the membrane by an unknown mechanism. According to the second, simultaneous-targeting model, the small and the large subunits of hydrogenases first form a complex, which is followed by the processing of the large subunit and then by membrane targeting of the complex by virtue of the signal sequence of the small subunit.

Accumulating evidence supports the second model. An interdependence of the subunits in the translocation of NiFe-hydrogenases was first described by Van Dongen et al. (1988). It was then confirmed by the study of the biosynthesis of NiFe-hydrogenases from *E. coli* (Menon et al. 1991, 1994), *Alcaligenes eutrophus* (Bernhard et al. 1996) and *Desulfovibrio gigas* (Rousset et al. 1998). Recently it was clearly shown that, unlike most secreted proteins, the signal peptide itself of the small subunit of hydrogenase 2 of *E. coli* is not sufficient for membrane targeting and translocation (Rodrigue et al. 1999). The presence of a truncated large subunit inhibits membrane targeting of the small subunit that accumulates in the cytoplasm as a precursor and that is also found in the aggre-

gated material. In addition, membrane targeting of the small subunit not only depends on the presence of the large subunit, but also on the nickel incorporation into, and the concomitant processing of, the large subunit. Reciprocally, the small subunit is required both for the membrane targeting of the large subunit and for nickel acquisition (Rodrigue et al. 1999), and the substitution of the twin-arginine motif of the signal peptide of the small subunit results in the cytoplasmic accumulation of both the small and the large subunits of the NiFe-hydrogenase from *Wolinella succinogenes* (Gross et al. 1999). Further investigation confirmed that the small and large subunits of hydrogenase 2 could be cross-linked in vitro in the cytoplasmic fraction, which suggests the formation of an enzyme complex prior to the membrane targeting. The most convincing results were obtained by substituting the targeting signal of the small subunit of hydrogenase 2. When the twin-arginine signal peptide of the small subunit was replaced by the typical Sec signal peptide of PelB, the large subunit of hydrogenase 2 of *E. coli* was mistargeted to the Sec machinery and was stuck in the SecYEG translocation channel (Rodrigue et al. 1999). Therefore, under these conditions it is the signal peptide of the small subunit that determines whether the large subunit follows the Sec or the Mtt (Tat) pathway.

Collectively, these results suggest the following scenario of a hitchhiker model for the cotranslocation of hydrogenase 2 of *E. coli* (Fig. 1). After translation of the *hybO* and *hybC* genes, the small subunit is protected by a chaperon protein from being targeted to the membrane and from misfolding and aggregation. It would also be involved in the formation of Fe-S clusters of the small subunits. The chaperon would be associated with the small subunit until the large subunit enters the complex and interacts with the small subunit. This protection is crucial for the stability of the small subunit, since two ORFs separate *hybO* (encoding the small subunit) from *hybC* (encoding the large subunit), implying an important lapse of time between the translation of the two genes. At this stage, the same chaperon protein, and other helper proteins, would bind to the complex, including the C-terminal extension segment, and then catalyze the incorporation of nickel and iron, thus leading to the formation of the metal center. This process might also be correlated with the assembly of the Fe-S clusters. Then the C-terminal extension sequence is cleaved, triggering the conformational change of the complex, and the twin-arginine signal peptide would become accessible. A cytoplasmic targeting factor would recognize the signal peptide and direct the complex to the Mtt (Tat) pathway. The enzyme complex is translocated independently of the Sec machinery across the cytoplasmic membrane through the Mtt (Tat) system (Bogsch et al. 1998; Chanal et al. 1998; Sargent et al. 1998b; Rodrigue et al. 1999). The signal peptide is cleaved and the complex remains attached to the membrane by the C-terminal anchor sequence of the small subunit (Fig. 1).

Twin-arginine, signal peptide-dependent protein translocation pathway

The twin-arginine signal peptide was originally found in periplasmic and membrane-bound hydrogenases (Voordouw 1992; Wu and Mandrand 1993). It was first reported that the twin-arginine signal peptide of the NiFe-hydrogenase of *D. vulgaris* is capable of exporting β-lactamase into the periplasm of *E. coli* (Nivière et al. 1992). Most

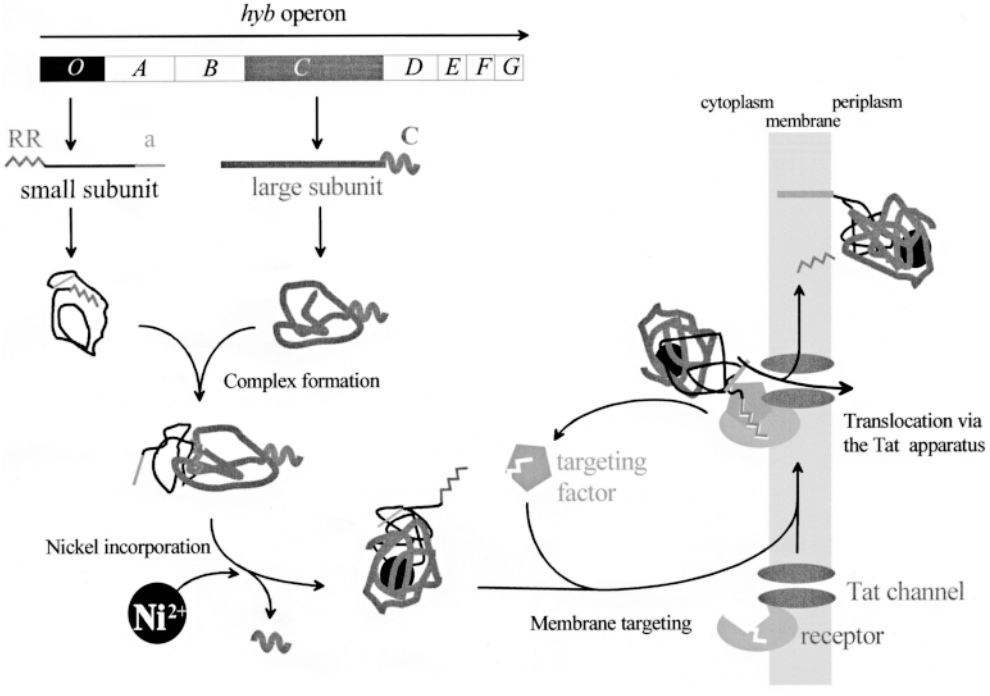

Fig. 1 Hitchhiker cotranslocation of the large subunit with the small subunit of NiFe-hydrogenase. The twin-arginine signal peptide is indicated by *RR*, the C-terminal anchor sequence of the small subunit of hydrogenase 2 by *a*, the C-terminal extension sequence of the large subunit by *C*

Table 1 Characteristics of proteins that can be translocated by the bacterial Mtt (Tat) pathway

Proteins	Origin	Redox cofactor	Reference[a]
Hydrogenase 1	*Escherichia coli*	Nickel-iron	1–3
Hydrogenase 2	*E. coli*	Nickel-iron	1–5
Hydrogenase	*Wolinella succinogenes*	Nickel-iron	6
TMAO reductase	*E. coli*	Molybdo-cofactor	1–5, 7
DMSO reductase	*E. coli*	Molybdo-cofactor	1–3, 7
Formate dehydrogenase	*E.a coli*	Molybdo-cofactor	1–3
Nitrate reductase	*E. coli*	Molybdo-cofactor	7
Yack	*E. coli*	Four copper ions	3
Glucose-fructose oxidoreducatse	*Zymomonas mobilis*	NADP	8
SufI	*E. coli*	None	3
β-Lactamase	*E. coli*	None	9
P2 Lep	*E. coli*	None	10
23 kDa protein	Thylakoids	None	2

[a]References: *1* Sargent et al. (1998b), *2* Bogsch et al. (1998), *3* Sargent et al. (1999), *4* Chanal et al. (1998), *5* Rodrigue et al. (1999), *6* Gross and Kröger (1999), *7* Weiner et al. (1998), *8* Halbig et al. (1999), *9* Nivière et al. (1992), *10* Cristobal et al. (1999)

importantly, the twin-arginine motif was found to be essential for the translocation. The twin-arginine signal peptide was then described as being conserved among various classes of bacterial enzymes containing redox cofactors, and it was proposed that it directs these enzymes to a particular Sec-independent protein translocation pathway (Berks 1996). To date, the translocation of at least seven bacterial enzymes carrying three types of redox cofactors has been shown to use this novel pathway (Table 1). In addition, the twin-arginine signal peptide is capable of directing the translocation of other polypeptides lacking cofactor, which might fold too quickly to be handled by the Sec pathway (Table 1 and our unpublished data).

Components of this novel pathway were recently identified (Settles et al. 1997; Bogsch et al. 1998; Chanal et al. 1998; Santini et al. 1998; Sargent et al. 1998b; Weiner et al. 1998; Rodrigue et al. 1999), and the system was designated first as membrane targeting and translocation (Mtt; Weiner et al. 1998) and then as a twin-arginine translocation (Tat; Sargent et al. 1998b) pathway. Genes encoding these components are present in major groups of bacteria, in mitochondria and in chloroplasts. In *E. coli*, genes encoding five Mtt/Tat components are located at two loci: *tatE* at 14 min and *tatABCD* at 86 min (Fig. 2; Settles et al. 1997; Sargent et al. 1998b; Weiner et al. 1998). TatE and TatA show about 50% sequence identity at the protein level, and they share about 25% sequence identity with TatB. The homologous region encompasses their N-terminal transmembrane segment and an adjacent amphipathic structure (Chanal et al. 1998). Their C-terminal domains are less well-conserved. A given mutation of the genes may affect the translocation of various enzymes to a differing extent (Fig. 2). For example, the depletion of *tatE* has very little effect on the translocation of hydrogenase 2 and no effect on that of hydrogenase 1 of *E. coli* (Sargent et al. 1998b). The removal of *tatA* slightly affects the translocation of both hydrogenases 1 and 2, whereas the double *tatA-tatE* deletion increases this effect considerably. The insertion mutation in the *tatB* gene severely blocks the translocation of TMAO reductase, but has only a slight effect on the translocation of hydrogenase 2 (Chanal et al. 1998). Considering that deletion of these genes affects the translocation of various enzymes differently, we proposed that TatA/B/E function as receptors for different substrates. In contrast, TatC contains six transmembrane segments and its depletion completely abolishes translocation

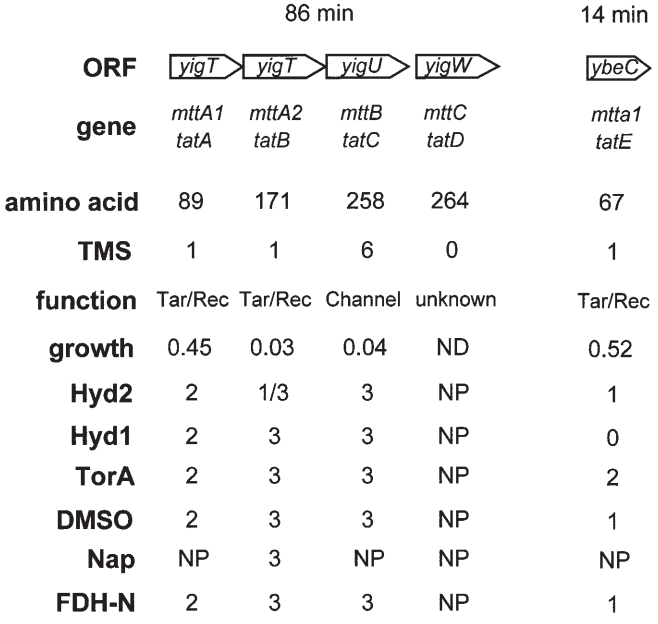

Fig. 2 Essential characteristics of the genes encoding the Mtt (Tat) components of *Escherichia coli*. Two loci, located at 86 min (*yig*) and 14 min (*ybe*), encode five potential Mtt components. Their corresponding ORF, *mtt* and *tat* nomenclatures are indicated. The sizes (in number of amino acids) of the encoded polypeptides and predicted transmembrane segments (*TMS*) are indicated. These components may be involved in membrane targeting (*Tar*), reception (*Rec*) or channel formation (see text). The phenotypes of the mutants are summarized. *ND* Not determined, *NP* not published. The corresponding mutants were grown anaerobically in minimal TMAO-glycerol medium after 24-h incubation at 37 °C; the optical density measured at 600 nm (our non-published results) is given. The value of the wild-type parental strain was 0.38. The effect of the mutations on the translocation of various enzymes was compiled from Bogsch et al. (1998), Chanal et al. (1998), Santini et al. (1998), Sargent et al. (1998b, 1999), Weiner et al. (1998), and Rodrigue et al. (1999). *0* No effect, *1* slight effect, *2* medium effect, *3* severe effect (for details see text)

of all the enzymes analyzed (Bogsch et al. 1998). Therefore, TatC may form the core structure of the Tat translocon. It should be noted that the *tatB* mutation was recently reported to completely interrupt the translocation of both hydrogenase 1 and hydrogenase 2, and TatB may function to stabilize TatC complexes (Sargent et al. 1999). The discrepancy in the effect of the *tatB* mutation on the translocation of hydrogenase 2 might be due to the difference in the growth conditions used. *tatD* is transcribed together with *tatABC* (Sargent et al. 1998b; Weiner et al. 1998), which leads to the assumption that TatD is a component of the Tat pathway. However, involvement of TatD and two other homologous soluble proteins in protein translocation has not been demonstrated.

A so-called ΔpH-driven pathway is used in protein import into the thylakoid lumen of chloroplasts and shares characteristics with the bacterial Mtt (Tat) pathway (Settles and Martienssen 1998). It operates independently of the Sec system, is energized by proton motive force and is capable of translocating proteins in a folded conformation. Substrates of the ΔpH-driven pathway and the Mtt (Tat) pathway contain similar twin-arginine signal peptides, and, most importantly, their twin-arginine signal peptides are exchangeable (Mori and Cline 1998). Another eukaryotic protein translocation pathway also shows mechanistic similarity to the translocation of hydrogenases. This system can effectively import polypeptides lacking signal sequence into the peroxisomal matrix in a "piggyback" fashion on other polypeptides containing signal sequences (Rachubinski and Subramani 1995). Components common to both pathways have not yet been identified.

Despite the considerable progress in our knowledge regarding hydrogenase translocation, its elucidation is still in a state of infancy and some essential questions remain unanswered. How are hydrogenases targeted to the membrane? How do bacterial cells distinguish a precursor lacking nickel from the mature enzyme competent for translocation? How does such a large enzyme cross the membrane? Seeking answers to these questions will be one of the major research directions in the study of hydrogenases in the next decade. Among the various biochemical approaches, we propose copurification of targeting factor(s) with the twin-arginine signal peptide, which is fused to a carrier protein to facilitate detection and purification. Immunocoprecipitation represents an alternative approach for this purpose. Truncation of either the small or the large subunit might allow determination of the "inhibitory" domain that prohibits translocation of the precursor lacking nickel. With respect to genetic approaches, two-hybrid experiments might elucidate the interaction between hydrogenase and different Tat components. Such an interaction might also be revealed by the suppression approach that has been very successfully used in the study of the Sec machinery.

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