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A universal system for the transport of redox proteins: early roots and latest developments ☆

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Abstract

The transport of proteins binding redox cofactors across a biological membrane is complicated by the fact that insertion of the redox cofactor is often a cytoplasmic process. These cytoplasmically assembled redox proteins must thus be transported in partially or completely folded form. The need for a special transport system for redox proteins was first recognized for periplasmic hydrogenases in Gram-negative bacteria. These enzymes, which catalyze the reaction $H_2 \leftrightarrow 2H^+ + 2e$, are composed of a large and a small subunit. Only the small subunit has an unusually long signal sequence of 30–50 amino acid residues, characterized by a conserved motif (S/T)–R–R–x–F–L–K at the N-terminus. This sequence directs export of the large and small subunit complex to the periplasm. Sequencing of microbial genes and genomes has shown that signal sequences with this conserved motif, now referred to as twin-arginine leaders, occur ubiquitously and export different classes of redox proteins, containing iron sulfur clusters, molybdopterin cofactors, polynuclear copper sites or flavin adenine dinucleotide. Mutations in an *Escherichia coli* operon referred to as *mtt* (membrane targeting and translocation) or *tat* (twin arginine translocation) are pleiotropic, i.e. these prevent the expression of a variety of periplasmic oxido-reductases in functional form. The Mtt or Tat pathway is distinct from the well-known Sec pathway and occurs ubiquitously in prokaryotes. The fact that its component proteins share sequence homology with proteins of the ΔpH pathway for protein transport associated with chloroplast thylakoid assembly, illustrates the universal nature of this novel protein translocation system.

Keywords: Hydrogenase; Transport of folded proteins; Twin-arginine leader; Periplasmic oxido-reductases; Thylakoid assembly; Redox prosthetic groups

☆ Dedicated to Heini Eisenberg.

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1. Introduction

It gives me great pleasure to write this article as a contribution to the Symposium on the Thermodynamics and Structure of Biological Macromolecules, held on 11 February 2000 in New Orleans to honor the contributions made to the field by Henryk Eisenberg. I worked in Heini's laboratory at the Polymer Department of The Weizmann Institute of Science in Rehovot, Israel as a National Research Council of Canada postdoctoral fellow in 1976 and returned several times on short visits sponsored by EMBO. The project suggested by Heini was to produce large amounts of ColE1 plasmid DNA and determine its physical properties in solution. With Dorit Kalif I started a small plasmid production factory and we obtained ample material for this project. This allowed us to determine key physical properties, such as the persistence length of the DNA, in the absence of polydispersity [1]. We also collected exciting biophysical data on the binding of histones to ColE1 plasmid DNA and nucleosome core particles [2,3]. These were happy and productive times of which both Hansje and I have fond memories. Heini greatly contributed to my formation as a scholar and has been very supportive of my career long after I left Israel.

Research on the topic of this paper started in 1985, when the genes for a periplasmic hydrogenase from the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough were cloned and sequenced [4,5]. This hydrogenase, referred to as an Fe-only hydrogenase because it contains only iron–sulfur clusters as prosthetic groups, was thought to reside in the periplasm of *D. vulgaris*. Sequencing indicated two subunits HydA and HydB of M_r 46 and 13.5 kDa, respectively. However, it was not at all clear from the translated nucleic acid sequence how this enzyme was transported to the periplasm, as typical Sec-dependent N-terminal signal sequences were missing. A first glimpse that an unusual translocation mechanism might be used for hydrogenase export came from the work of Prickril et al. [6], who found that an N-terminal sequence of 34 amino acid residues was cleaved from the small subunit (Fig. 1: DvHHydB), whereas the large subunit lacked an N-terminal signal sequence [5,6]. The notion of a unique mechanism for hydrogenase transport, suggested in the following years by the sequencing of more hydrogenase operons and biochemical studies of hydrogenase assembly and transport, was accepted among workers in this field in the early nineties. However, the general nature of this transport system was not realized. More recently, it has become clear that the unusual mechanism, proposed for hydrogenase transport on the basis of these early studies, is much more generally used for transport of a wide variety of redox proteins from the cytoplasm, where they are synthesized and (partially) assembled, to the periplasm. These early and more recent studies will be reviewed here.

2. Signal sequences of periplasmic hydrogenases

Periplasmic Ni–Fe hydrogenases are more widespread in Gram-negative bacteria than the Fe-only enzymes. They also consist of a large (L, 60 kDa) and a small (S, 30 kDa) subunit, that combine to a 90-kDa LS heterodimer. These enzymes occur as soluble periplasmic enzymes (Fig. 1: Dg-HynB to Df-HynB) or as membrane-bound periplasmic enzymes (Fig. 1: Rl-HupS to Tr-HupS). In the latter case the small subunit has a C-terminal hydrophobic extension that serves to anchor the LS heterodimer to the membrane. Structural studies of the Ni–Fe hydrogenases from *D. gigas* and *D. vulgaris* have shown that the Ni-containing active center is located in the large subunit, whereas the small subunit has iron–sulfur clusters for its electron transfer function [7,8]. The nucleotide sequences of the Ni–Fe hydrogenase genes from *D. gigas* and *D. baculatus*, indicated no sequence homology with Fe-only hydrogenase [9,10]. The only significant sequence similarity was at the N-terminus of the small subunits, which shared a sequence RRxFxK (Fig. 1: Dg-HynB and Db-HysB). Soon many other gene translated sequences of periplasmic Ni–Fe hydrogenases appeared. The presence of a long signal sequence at the N-terminus of the small subunit and the lack of an N-terminal signal sequence in the large subunit was found to be a universal

Fig. 1. Comparison of selected Sec-dependent signal peptides (Ec-Bla to Dv-HmcA) with twin-arginine signal peptides that function in hydrogenase transport (Dg-HynB to DvMHydB). The twin-arginine sequences displayed are a subset of a larger compilation provided elsewhere [24]. Strictly conserved residues are indicated in bold. The order of these sequences from top to bottom is the same as in the dendrogram in Fig. 2. Sec-dependent signal sequences are for β-lactamase from *E. coli* (Ec-Bla), cytochome c_3, cytochrome c_{553}, and HmcA from *Desulfovibrio vulgaris* Hildenborough (Dv-Cyc, Dv-Cyf and Dv-HmcA, respectively). Listed are twin-arginine signal sequences at the N-terminus of the small subunit of Ni–Fe hydrogenase from *D. gigas* (Dg-HynB), *D. vulgaris* (Dv-HynB), *D. fructosovorans* (Dv-HynB), *Rhizobium leguminosarum* (Rl-HupS), *Bradyrhizobium japonicum* (Bj-HupS), *E. coli* (Ec-HyA), *Citrobacter freundii* (Cf-HyA), *Rhodobacter capsulatus* (Rc-HupS), *Rhodobacter sphaeroides* (Rs-HupS), *Pseudomonas hydrogenovora* (Ph-HupS), *Azotobacter vinelandii* (Av-HoxK), *Alcaligenes eutrophus* (Ae-HoxK), *Rhodocyclus gelatinosus* (Rg-HupS), *Alcaligenes hydrogenophilus* (Ah-HupS), *Thiocapsa roseopersicina* (Tr-HupS), *D. baculatus* (Dd-HysB; this enzyme also contains selenium), and *Wolinella succinogenes* (Ws-HydA). Also listed are twin-arginine signal sequences at the N-terminus of the small subunit of Fe-only hydrogenase from *D. vulgaris* Hildenborough (DvHHydB) and *D. vulgaris* Monticello (DvMHydB).

feature of these enzymes. All small subunit signal sequences had the conserved sequence RRxFxK near the N-terminus (Fig. 1), suggesting an unusual but conserved export mechanism. Phylogenetic analysis of these hydrogenase signal sequences indicates separate clades for the signal sequences of the Ni–Fe and Fe-only hydrogenases (Fig. 2). Within the Ni–Fe hydrogenase signal sequence clade the soluble and membrane-bound enzymes form distinct groups (Fig. 2). The signal sequences of the selenium-containing Ni–Fe hydrogenase from *Desulfovibrio baculatus* (Figs. 1 and 2: Db-HysB) and of the Ni–Fe hydrogenase from *Wolinella succinogenes* (Figs. 1 and 2: Ws-HydA) are distinct within the group of Ni–Fe hydrogenase signal sequences (Fig. 2). Study of heterologous expression of Fe-only hydrogenase in *E. coli* indicated that this enzyme is assembled only partially and is, perhaps as a result, exported with poor efficiency [11,12]. Limited export was dependent on small subunit processing and was completely abolished if only the large or only the small subunit was expressed in *E. coli* [12]. The translocation model that emerged from these studies was that: (i) the small subunit precursor binds to the membrane through its signal se-

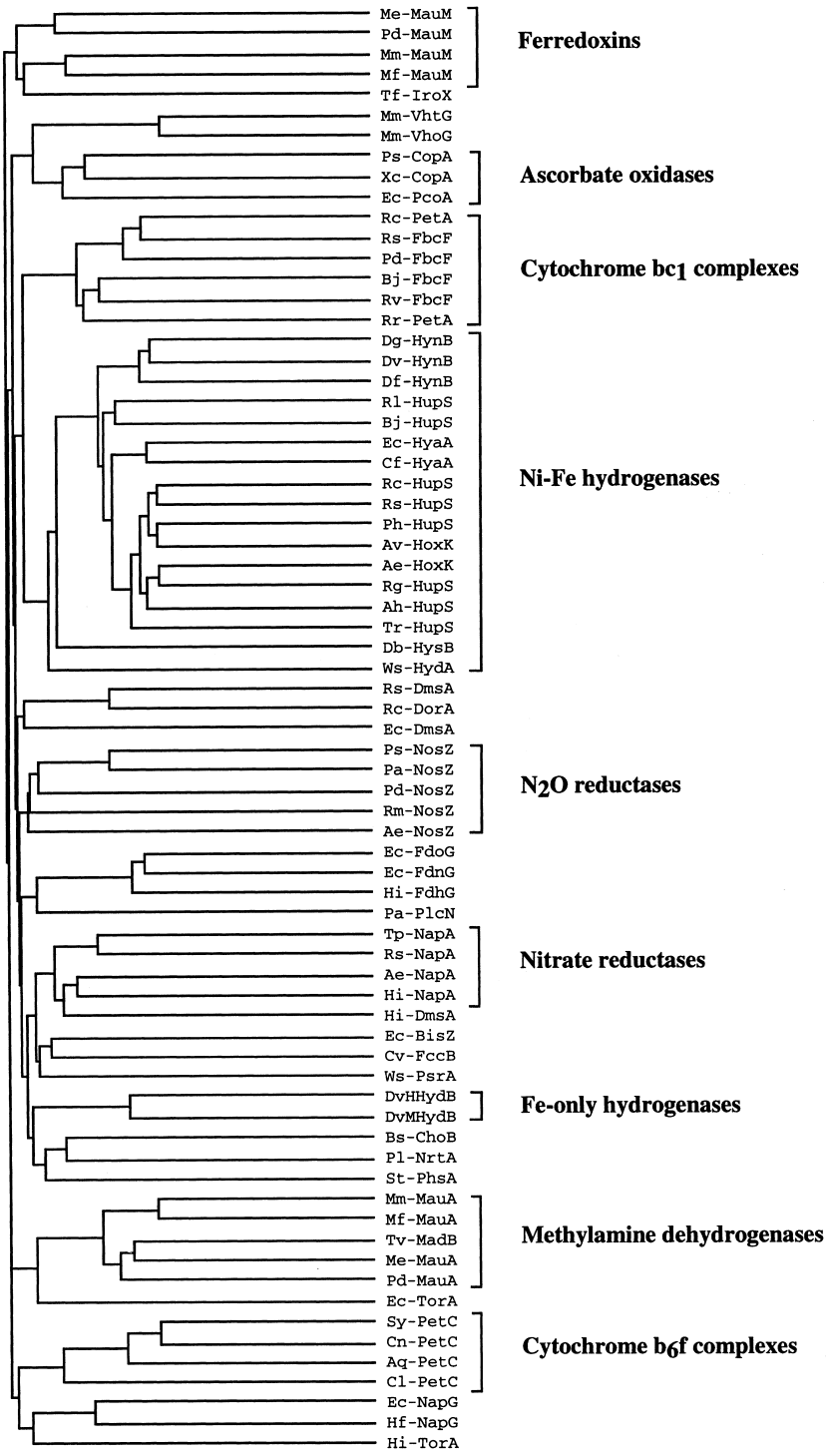
Me-MauM
Pd-MauM
Mm-MauM
Mf-MauM
Tf-IroX
Mm-VhtG
Mm-VhoG
Ps-CopA
Xc-CopA
Ec-PcoA
Rc-PetA
Rs-FbcF
Pd-FbcF
Bj-FbcF
Rv-FbcF
Rr-PetA
Dg-HynB
Dv-HynB
Df-HynB
Rl-HupS
Bj-HupS
Ec-HyaA
Cf-HyaA
Rc-HupS
Rs-HupS
Ph-HupS
Av-HoxK
Ae-HoxK
Rg-HupS
Ah-HupS
Tr-HupS
Db-HysB
Ws-HydA
Rs-DmsA
Rc-DorA
Ec-DmsA
Ps-NosZ
Pa-NosZ
Pd-NosZ
Rm-NosZ
Ae-NosZ
Ec-FdoG
Ec-FdnG
Hi-FdhG
Pa-PlcN
Tp-NapA
Rs-NapA
Ae-NapA
Hi-NapA
Hi-DmsA
Ec-BisZ
Cv-FccB
Ws-PsrA
DvHHydB
DvMHydB
Bs-ChoB
Pl-NrtA
St-PhsA
Mm-MauA
Mf-MauA
Tv-MadB
Me-MauA
Pd-MauA
Ec-TorA
Sy-PetC
Cn-PetC
Aq-PetC
Cl-PetC
Ec-NapG
Hf-NapG
Hi-TorA
Ferredoxins
Ascorbate oxidases
Cytochrome bc1 complexes
Ni-Fe hydrogenases
N2O reductases
Nitrate reductases
Fe-only hydrogenases
Methylamine dehydrogenases
Cytochrome b6f complexes

Fig. 2.

quence; (ii) associates with completely or partially folded large subunit; and (iii) the complex is then translocated and proteolytically processed [12,13]. Interestingly, Fe-only hydrogenases also occur as cytoplasmic enzymes in gram positive Clostridia, where they function primarily in hydrogen production. CpI, the enzyme from *Clostridium pasteurianum* is a single polypeptide of 60 kDa, containing an active site H cluster and five FeS clusters [14]. The N-terminal part of this protein is homologous to the HydA subunit and the C-terminal part to the HydB subunit of the periplasmic Fe-only hydrogenase from *D. vulgaris* [15]. Assuming that the periplasmic enzyme evolved from a cytoplasmic precursor, it appears that the signal peptide was not inserted at the N-terminus, but near the C-terminal end of the cytoplasmic protein [15]. This suggests that in *Desulfovibrio,* there is a finely tuned interplay between synthesis, folding, cofactor insertion and translocation that ensures that catalytically active Fe-only hydrogenase appears exclusively in the periplasm. The nature of the complex Ni–Fe hydrogenase signal peptide was explored by Nivière et al. [16], who studied the translocation of a chimeric protein in which the signal peptide of the small subunit of Ni–Fe hydrogenase from *D. vulgaris* (Fig. 1: Dv-HynB) was fused to β-lactamase. The enzyme β-lactamase is normally translocated to the *E. coli* periplasm through the Sec pathway. The Sec-dependent signal sequence (Fig. 1: Ec-Bla) was replaced by the hydrogenase signal sequence in the chimeric protein. It appeared that the fusion protein was more efficiently exported to the *E. coli* periplasm under anaerobic conditions than under aerobic conditions. Export was critically important on the integrity of the hydrogenase signal peptide consensus sequence, i.e. mutagenesis of the first Arg of this sequence to other residues (Glu, Val, Met, but also Lys) abolished export. These results suggested that the chimeric protein was translocated with the help of a protein that specifically recognizes the hydrogenase signal peptide consensus sequence and that is present in *E. coli* primarily under anaerobic conditions [16]. This last feature was not considered surprising, as *E. coli* expresses Ni–Fe hydrogenases 1 and 2 under anaerobic conditions (Fig. 1: Ec-HyaA is the signal sequence for Ni–Fe hydrogenase 1) Similar results to those reported by van Dongen et al. [12] were recently reported for the export of *E. coli* Ni–Fe hydrogenase 2 [17]. The small subunit precursor or the large subunit accumulated in the *E. coli* cytoplasm when either was expressed in the absence of the other subunit, suggesting that the large subunit is exported as part of the LS complex through recognition of the small subunit signal peptide. Rodigue et al. referred to this as a hitchhiker mechanism [17]. Gross et al. [18] reported a site-directed mutagenesis study of the twin-arginine motif of the signal peptide of *Wolinella succinogenes* Ni–Fe hydrogenase. This enzyme is encoded by three genes organized in the *hydABC* operon. The *hydAB* genes encode a membrane-bound Ni–Fe hydrogenase. HydA represents the N-terminal, twin-arginine signal peptide containing small subunit (Fig. 1: Ws-HydA) and HydB the nickel-containing catalytic large subunit. The HydAB heterodimer resides in the periplasm, where it binds to HydC, which is a *b*-type heme containing, integral membrane cytochrome. Replacement of either of the two conserved arginines of the RRDFMK consensus sequence by glutamine residues abolished export of the heterodimer. The HydC protein appeared to be correctly assembled into the membrane, but the HydAB heterodimer accumulated on the inside face of the cytoplasmic membrane. HydA was not processed, but HydB apparently contained Ni because it exhibited H_2-dependent benzylviologen reductase activity. The organism could not grow

Fig. 2. Dendrogram of twin-arginine signal peptides. The sequences analyzed are a subset of the listing in [24]. Only sequences extending from position −4 to +24 relative to the twin-arginine consensus sequence (positions 0 and 1) were used for the analysis. The dendrogram was obtained with the PileUp program of the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, WI). The horizontal axis reflects the degree of relatedness of the sequences. Twin arginine sequences for Ni–Fe and Fe-only hydrogenases are explained in the legend to Fig. 1. References to all other sequences are given in Berks [24]

with H_2 as the electron donor for the anaerobic respiratory chain, indicating that the misoriented heterodimer could not donate electrons to HydC.

3. Twin-arginine leaders for redox protein export

Following the discovery that hydrogenase signal peptides direct export of (partially) folded and assembled proteins through a unique and novel pathway, it became apparent that this pathway was much more universally used for redox protein export. Bokrantz et al. [19] found that FdhA, the 101 kDa catalytic subunit of *W. succinogenes* formate dehydrogenase, which needs insertion of molybdopterin guanine dinucleotide (MGD) for activity, has an N-terminal, hydrogenase-type signal sequence. Similar to NiFe-hydrogenase from the same organism, periplasmic formate dehydrogenase consists of FdhA, B and C subunits. FdhB (32 kDa, four Fe_4S_4 clusters) is an electron-transferring subunit, that lacks a signal sequence. FdhC (32 kDa) is a *b*-type heme containing, integral membrane cytochrome, similar to HydC. Berg et al. [20] showed that FdnG, the catalytic subunit of nitrate-inducible formate dehydrogenase from *E. coli* also contains a hydrogenase-type signal sequence. This enzyme consists of FdnG, H and I subunits, which are homologous to FdhA, B, and C, respectively. These enzymes are likely to be topologically arranged as a periplasmic heterodimer (FdhAB, FdnGH) bound to the heme-containing, integral membrane subunit (FdhC or FdnI, respectively). Thus, FdhB and FdnH may also be exported to the periplasm by a hitchhiker mechanism, but contrary to Ni–Fe hydrogenase, the signal peptide is on the large, not on the small subunit.

A hydrogenase-type signal peptide was also found at the N-terminus of HmcB (40 kDa, four Fe_4S_4 clusters), the electron-transferring subunit of the Hmc complex [21–23]. This membrane-bound complex catalyzes electron transport from the periplasmic, hydrogen oxidation pathway to the cytoplasmic sulfate reduction pathway in sulfate-reducing bacteria of the genus *Desulfovibrio*. It consists of HmcA (55 kDa), a periplasmic cytochrome containing 16 covalently bound *c*-type hemes, HmcB (homologous to FdhB and FdnH), HmcC (43 kDa), HmcD (6 kDa), and HmcE (25 kDa), three integral membrane proteins possibly containing *b*-type heme, and HmcF (53 kDa), a cytoplasmic redox protein containing iron–sulfur clusters. In Gram-negative bacteria *c*-type cytochromes are located exclusively in the periplasm. However, because heme is also exported and is covalently connected with the apoprotein in the periplasm, *c*-type cytochrome polypeptides are exported through a conventional Sec-dependent leader sequence. *D. vulgaris* has three periplasmic *c*-type cytochromes, cytochrome c_{553}, cytochrome c_3 and HmcA, containing 1, 4 and 16 hemes, respectively. Their signal sequences, indicated in Fig. 1 as Dv-Cyf, Dv-Cyc and Dv-HmcA, do not contain the RRxFxK consensus sequence. Thus, in assembling the Hmc complex, HmcA is exported through a Sec-dependent leader and HmcB through a hydrogenase-type signal sequence.

Compelling evidence for the existence of a common export pathway for proteins binding redox cofactors (with the exception of *c*-type cytochromes) was presented by Berks [24], who searched the rapidly expanding sequence databases for the presence of double arginine leader sequences. Berks provided a compilation of 90 proven or putative signal sequences as an addendum to his paper. Although all contained a double arginine sequence, other elements of the previously derived consensus (Fig. 1) were less strictly conserved. Berks redefined the consensus sequence as (S/T)–R–R–x–F–L–K. The percentage frequency of occurrence of these residues was (54/21)–100–100–x–82–53–61. Because the two arginines represent the only strictly conserved sequence element, Berks renamed the hydrogenase-type signal sequences, as double arginine or twin-arginine signal sequences. The sequence relatedness of a subset of these is presented as a dendrogram in Fig. 2. The sequences group according to the function of the protein they export, not according to the organism in which they reside, i.e. the twin-arginine leaders of all Ni–Fe hydrogenases (the largest group in Fig. 2) group together. In contrast, *E. coli* twin-arginine leaders for a variety of redox proteins (Fig. 2: Ec-PcoA, Ec-HyaA, Ec-DmsA, Ec-FdoG,

Ec-FdnG, Ec-BisZ, Ec-TorA and Ec-NapG) are distributed all over the dendrogram. It appears, therefore, that twin-arginine leader sequences have evolved together with the redox protein that they help to export. Thus, signal peptide swapping experiments, as described by Nivière et al. [16] are bound to lead to a significant decrease in export efficiency, as was indeed observed in that study. Not all sequences group together functionally. For instance, the large class of periplasmic proteins binding an MGD cofactor [24] includes sequences for soluble periplasmic enzymes (Fig. 2: Ec-TorA, Hi-TorA, Rs-DmsA, Rc-DorA and Ec-BisZ), for soluble, periplasmic nitrate reductases (Fig. 2: Tp-NapA, Rs-NapA, Ar-NapA and Hi-NapA) and for periplasmic membrane-bound redox protein complexes (Fig. 2: Ec-FdoG, Ec-FdnG, Hi-FdhG, Ws-FdhA, Ec-DmsA, Hi-DmsA, Ws-PsrA and St-PhsA). The sequences in this class do not all group together, but form several distinct clades in the lower half of the dendrogram (Fig. 2).

The importance of the twin-arginine motif for export of these redox proteins has been proven by site-directed mutagenesis studies in some cases. Dreusch et al. [25] reported that an Arg-to-Asp mutation of the first arginine of the conserved pair in the signal sequence of nitrous oxide reductase from *Pseudomonas stutzeri*, caused accumulation of the enzyme in the cytoplasm in an unprocessed form. This enzyme requires a correctly assembled bi-nuclear Cu_A site for activity. The cytoplasmic form was found to be devoid of copper, suggesting that copper insertion is either periplasmic or takes place during transport of nitrous oxide reductase to the periplasm.

4. Components of the *mtt* or *tat* translocation system

Mutations in genes for proteins active in twin-arginine translocation may be expected to be pleiotropic. In some microorganisms such mutations may nevertheless be viable. For instance in *E. coli* periplasmic reductases are required only for anaerobic growth. Multiple defects in anaerobic respiration would not impair growth under aerobic conditions. The components of the twin-arginine translocation system were identified through sequence homology with chloroplast counterparts [26,27] and through complementation analysis of an *E. coli* mutant with multiple defects in anaerobic metabolism [28]. Protein import from the chloroplast stroma to lumen is topologically equivalent to export from the cytoplasm to the periplasm in Gram-negative bacteria. In chloroplast thylakoids two distinctly different import pathways have been identified, the Sec and ΔpH pathways. The Sec pathway is similar to that in bacteria, which have a membrane-bound complex consisting of SecA, E, G, and Y components. This complex drives transport of proteins in unfolded form by a threading mechanism through ATP hydrolysis by SecA. Sec-dependent transport can be inhibited specifically by azide, both in bacteria and chloroplasts. The second transport pathway in chloroplasts, the ΔpH pathway, is inhibited by the ionophores nigericin and valinomycin that dissipate the proton gradient across the thylakoid membrane. It had been established that proteins transported through the ΔpH pathway have a signal peptide with an essential twin-arginine motif. Moreover, mutations in maize causing defects in chloroplast electron transport and recognizable by virtue of their high chlorophyll fluorescence (*hcf*) have been described. One of these (*hcf*106) specifically affects ΔpH-driven protein transport. Settles et al. cloned the *hcf*106 gene and showed that it is a receptor-like thylakoid membrane protein of 243 amino acids with a single transmembrane domain [26]. Homologs of Hcf106 were found in many bacterial genomes, e.g. those of *E. coli*, *Haemophilus influenzae*, *Bacillus subtilis*, and *Synechocystis* PCC6803. The homology included the membrane-spanning domain and an adjacent C-terminal stretch of 40 amino acids, predicted to be present in the bacterial cytoplasm. The bacterial homologs were found in an operon encoding several other genes that were expected to encode the pore for the twin-arginine translocation pathway. Settles et al. noted that experimental proof for this suggestion was already available, because

a mutation abolishing hydrogenase export in *Azotobacter chroococcum* mapped to the operon with the *hcf*106 homolog [26].

Weiner et al. identified the genes required for twin-arginine translocation independently by complementing *E. coli* D-43, a mutant generated from *E. coli* HB101 by chemical mutagenesis [28]. *E. coli* D-43 has multiple defects in anaerobic respiration. It can not grow on media containing dimethylsulfoxide (DMSO) or trimethylamine N-oxide (TMAO) as sole electron acceptor for the electron transport chain and has a reduced growth on media containing fumarate or nitrate as the electron acceptor. Complementation of this mutation pointed to the same operon as identified by Settles et al. [26]. Weiner et al. referred to this as the *mtt* operon (for membrane targeting and translocation) and indicated it to consist of the *mttA*, *B* and *C* genes, following resequencing of the locus. The specific nature of the protein transport defect in *E. coli* D-43 was elegantly demonstrated by comparing translocation of NapA and NrfA, the periplasmic nitrate and nitrite reductase, respectively, which are both required for dissimilatory reduction of nitrate to ammonia under anaerobic conditions. The former has an MGD-containing, catalytic subunit that is exported through a twin-arginine leader, whereas the latter is a *c*-type cytochrome exported through a Sec-dependent leader (see examples in Fig. 1). NapA was not exported to the periplasm of D-43, but localization of NrfA was normal [28]. Resequencing of the *mtt* operon region by Sargent et al. [27] indicated the presence of four rather than three genes, referred to as *tatA*, *B*, *C* and *D* (for twin-arginine translocation). A stop codon, apparently overlooked by Weiner et al. [28], split *mttA* into the *tatA* and *tatB* genes. Sargent et al. provided independent evidence for four genes in the operon by expression analysis. TatA (89 amino acids, 9.6 kDa) has a single transmembrane helix and is a homolog of maize chloroplast Hcf106. Settles et al. already reported that *E. coli* contains a second *hcf*106 homolog, the *ybeC* gene. This gene, renamed *tatE* by Sargent et al. [27], is present as a monocistronic transcriptional unit elsewhere in the chromosome. TatA and TatE share 50% amino acid sequence identity. TatB (171 amino acids, 18.4 kDa) has an N-terminal transmembrane helix, followed by a cytoplasmic domain. Interestingly, the transmembrane region of TatB is also homologous (25% amino acid identity) to that of TatA and TatE [29]. TatC (258 amino acids, 28.9 kDa; MttB of Weiner et al. [28]) is an integral membrane protein composed of 6 transmembrane helices, whereas TatD (264 amino acids, 29.5 kDa; MttC of Weiner et al. [28]) has a single N-terminal transmembrane helix with the rest of the protein being cytoplasmic. TatC (MttB) homologs are widespread in bacteria, mitochondria and chloroplasts. TatD (MttC) homologs are similarly widely distributed, although the involvement of TatD in twin-arginine translocation is uncertain, because a mutation in this gene appears to have no effect [30]. The ubiquitous distribution of *tat*-homologs and the demonstration that the bacterial twin-arginine and the chloroplast ΔpH pathway are structurally and functionally related indicates this protein transport pathway to be of universal importance.

The Tat-translocation complex in *E. coli* thus comprises three Hcf106 homologs (TatA, TatB and TatE), which all have a single transmembrane helix, and TatC an integral membrane protein with six transmembrane helices. The mutation in *E. coli* D-43 maps to the *tatB* gene and converts a critical Pro to a Leu. The work of Weiner et al. thus proved the critical importance of TatB in the twin-arginine translocation pathway. Sargent et al. constructed in frame deletion mutants of *tatA* and t*atE*. Both single mutations and a *tatA*, *tatE* double mutant were studied. Mislocalization of redox proteins exported through the twin arginine pathway was most severe in the double mutant, indicating that TatA and TatE can substitute for each other. In the double mutant TMAO reductase activity was completely absent from the periplasm, nitrate-inducible formate dehydrogenase accumulated as a cytoplasmic precursor and membrane-bound, periplasmic Ni–Fe hydrogenase 1 activity was completely absent. The *tatA, tatE* double mutation did not affect Sec-dependent protein export [27]. Chanal et al. [29] have suggested that TatA, TatB and TatE function independently of each other as a trimer in translocation of distinct sub-

sets of redox proteins, but that notion was disproved by the recent demonstration that an in frame deletion in *tatB* destabilized TatC [30]. These data indicate that TatB and TatC form a complex, that further interacts with TatA and TatE (the latter two being to some extent interchangable) that allows twin-arginine-dependent redox protein export in *E. coli*.

5. Conclusion

The idea that export of proteins binding complex redox cofactors is achieved through a specific pore, that allows transport of (partially) folded proteins was formulated approximately 10 years ago. The recent discovery of the *tat* (*mtt*) operon has provided strong evidence for the notion that this transport is though a single, conserved, membrane-bound protein complex. Prediction of the wide distribution and general nature of this translocation system was possible only because of the availability of whole genome sequences for many organisms. Interesting discoveries, such as the localization and nature of the twin arginine binding site that will further define the modus operandi of this system, may be anticipated in the future.

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