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Assembly of Tat-dependent [NiFe] hydrogenases: identification of precursor-binding accessory proteins

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Abstract The *Escherichia coli* twin-arginine translocation (Tat) system serves to export fully folded protein substrates across the bacterial cytoplasmic membrane. Respiratory [NiFe] hydrogenases are synthesised as precursors with twin-arginine signal peptides and transported as large, cofactor-containing, multi-subunit complexes by the Tat system. Cofactor insertion and assembly of [NiFe] hydrogenases requires coordination of networks of accessory proteins. In this work we utilise a bacterial two-hybrid assay to demonstrate protein–protein interactions between the uncharacterised chaperones HyaE and HybE with Tat signal peptide-bearing hydrogenase precursors. It is proposed that the chaperones act at a 'proofreading' stage in hydrogenase assembly and police the protein transport pathway preventing premature targeting of Tat-dependent hydrogenases.

Key words: Twin-arginine translocation protein transport system; Twin-arginine signal peptide; [NiFe] hydrogenase; Accessory protein; *Escherichia coli*

1. Introduction

Hydrogenase enzymes are widespread in prokaryotic and lower eukaryotic biological systems where they catalyse the reversible oxidation of molecular hydrogen to protons and electrons [1]. Hydrogenases are diverse in structure and can be classified according to cofactor content, thus [NiFe] hydrogenases, [Fe] hydrogenases, and metal-free hydrogenases have been described [1]. Of these, the model bacterium *Escherichia coli* contains only [NiFe] hydrogenases [1,2].

In *E. coli* respiratory hydrogen oxidation ('uptake') linked to quinone reduction is performed by [NiFe] hydrogenases-1 and -2 which are multi-subunit, membrane-bound, nickel-containing Fe-S proteins [1]. The bulk of these uptake enzymes (including the catalytic subunits containing the complex Ni-Fe-CO-2CN active sites) are exposed to the periplasmic space.

By analogy with other membrane-bound [NiFe] hydrogenases [1], hydrogenase-1 is probably a heterotrimeric enzyme consisting of a core heterodimer of an Fe-S cluster-binding β-subunit (HyaA), together with an α-subunit that binds the Ni-Fe active site cofactor (HyaB) [3] which associates with a third integral membrane cytochrome *b* γ-subunit (HyaC) to form the holoenzyme. The *E. coli* [NiFe] hydrogenase-2 isoenzyme is slightly different in structure from hydrogenase-1. The core catalytic dimer consists of the HybOC complex in which HybC is the α-subunit and HybO is the β-subunit [4]. The αβ-dimer associates with a periplasmically-oriented '16Fe' ferredoxin (HybA) and a cofactor-less integral membrane protein (HybB) to form a large tetrameric complex [5].

In order to facilitate transport of the core catalytic subunits to the periplasm uptake hydrogenase β-subunits are synthesised as precursors with cleavable N-terminal 'twin-arginine' signal peptides containing the conserved SRRxFLK amino acid motif (Fig. 1B; [6]). The twin-arginine motif has been shown to be essential for the transport of a diverse range of periplasmic proteins (reviewed in [7]) and, more specifically, is absolutely required for successful assembly of the uptake hydrogenases from *Ralstonia eutropha* and *Wolinella succinogenes* [8,9]. Twin-arginine signal peptides target precursor proteins to the membrane-embedded Tat (twin-arginine translocation) protein export system [7]. The Tat translocase has been shown to transport *fully folded* proteins across the energy-transducing inner membrane using energy provided by the transmembrane Δp [10]. Moreover, the Tat system has been shown to transport the prefolded and enzymatically active hydrogenase-2 αβ-dimer (HybOC) even though the twin-arginine signal peptide is located on only one of the subunits [11].

During the biosynthesis of uptake hydrogenases it is important that export of the enzymes is not performed, or even attempted, until all assembly processes have been fulfilled. It is possible, therefore, that the cell employs mechanisms to coordinate hydrogenase assembly and prevent wasteful export of malfolded or immature enzymes. Indeed, such 'proofreading' of Tat substrate protein 'maturity' (perhaps analogous to a measure of folded state) seems to operate as a two-tier system: Firstly, the folded state of a Tat substrate may be monitored at a relatively early stage by cytoplasmic chaperones. For example, biosynthesis of a subset of Tat-dependent enzymes involves the DmsD chaperone which binds to the Tat signal peptides of the dimethyl sulfoxide (DMSO) and trimethylamine *N*-oxide (TMAO) reductases [12], probably acting to mask the signal from the Tat machinery while simultaneously preventing premature folding during cofactor loading [13]. Secondly, folded state may be monitored at a later stage in the export process. For example, experiments involving fusions of a selection of *E. coli* Tat signal peptides to alkaline phosphatase (a reporter enzyme containing structural disulfide bridges) demonstrated that the Tat translocase will only trans-

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port passenger proteins that have attained at least a near-native conformation [14].

In this work we set out to identify putative Tat signal peptide-binding chaperones (or full-length precursor-binding proteins) using the [NiFe] hydrogenases of *E. coli* as a model. Uptake [NiFe] hydrogenases-1 and -2 are transported as preformed core αβ-dimers [11] and the [NiFe] cofactor must be correctly loaded into the α-subunits prior to dimerisation with the β-subunit partners [15]. Likewise Fe-S clusters must be assembled into the β-subunits prior to association with the α-subunits. Moreover, while the core hydrogenase adopts an $\alpha_1\beta_1$ stoichiometry prior to export [11], there is evidence that, following successful Tat transport, the uptake hydrogenases attain an higher-order $\alpha_2\beta_2$ conformation [2]. Paradoxically, therefore, the cell must ensure formation of the core αβ-heterodimer and also prevent assembly of the larger oligomers prior to transport. Consistent with these complicated assembly requirements, uptake hydrogenases are co-expressed with a number of dedicated accessory proteins. Hydrogenases-1 and -2 are encoded by two large genetic loci [4,16] (Fig. 1A). The functions of the seven non-structural genes encoded by the *hya* and *hyb* operons (Fig. 1A) have been characterised to variable degrees and most are involved in [NiFe] cofactor assembly [1,17]. Notably, however, the HyaE and HybE gene products (Fig. 1A) are of particular interest as there appear to be no homologous proteins required for the biosynthesis of cytoplasmic (i.e. non-exported) [NiFe] hydrogenases. The HyaE protein is a homologue of *R. eutropha* HoxO and *Rhizobium leguminosarum* HupG. Deletion of *hoxO* in *R. eutropha* led to complete loss of the uptake [NiFe] hydrogenase activity [8] pointing to a critical role for this gene in hydrogenase assembly. HybE is a homologue of *R. eutropha* HoxT and *R. leguminosarum* HupJ. Partial deletion of *hoxT* in *R. eutropha* resulted in an unusual phenotype in which the uptake hydrogenase was apparently still membrane targeted and enzymatically active with artificial electron acceptors, yet physiologically inactive [11]. Casalot and Rousset [17] and Dubini et al. [5] postulated that, due to their exclusive clustering with Tat-dependent hydrogenases, *hyaE* and *hybE* may have a role in maturation of the twin-arginine signal peptide-bearing β-subunits (Fig. 1B). Here, we address this issue directly by use of a bacterial two-hybrid system. We show that HyaE and HybE interact with the Tat signal peptide-bearing precursor forms of the hydrogenase β-subunits and a model by which these proteins act as molecular 'traffic police' during the Tat-dependent export of large multi-subunit [NiFe] hydrogenases is proposed.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli JM109 (Promega, UK) was used as the recipient strain for all plasmid constructions. The reporter strain for the two-hybrid assays was a derivative of *E. coli* K-12 strain MC1000 termed KS1 (F′ (*lacI*^{q}), *araD139*, Δ(*araA-leu*)*7697*, Δ(*lacI-Y*)*74*, *galE15*, *galK16*, λ-, *relA1*, *rpsL150*, *spoT1*, *e15-*, *attB*::p*lac*O_R*2-62*[Kan^R]) containing a chromosomal *lac* promoter derivative linked to the *lacZ* reporter gene and has been described previously [18].

Plasmid pBT ('bait'; Cm^R) encodes the λcI repressor and plasmid pTRG ('target'; Tet^R) encodes the α-subunit of *E. coli* RNA polymerase and are marketed as part of Stratagene Europe's Bacteriomatch® bacterial two-hybrid system. Genes of interest were amplified by polymerase chain reaction (PCR) and cloned downstream of, and in frame with, the reporter genes. All clones were sequenced to confirm fidelity of PCR and correct orientation of inserts.

2.2. Interaction assays

Expression of the fusion constructs on both reporter plasmids is under the control of *lacUV5* promoters. Upon expression of pBT and pTRG derivatives in the reporter strain KS1 the bacteriophage λcI protein binds to the λ operator (O_R2) upstream of the chromosomal reporter gene cassette. If the proteins of interest interact, DNA binding of the λcI portion of the fusion complex stabilises binding of the α-subunit of RNA polymerase to the test promoter and activates transcription of *lacZ* [19].

Strain KS1 was freshly transformed with pBT and pTRG derivatives. Single colonies were grown in 5 ml Luria–Bertani (LB) broth containing kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), tetracycline (15 μg/ml) and four increasing concentrations of isopropyl thiogalactose (IPTG) (0, 20, 50, and 100 μM final concentrations). Cultures were grown aerobically at 37°C for 16 h before whole cell assays of β-galactosidase activity were performed as described [19]. Assays were performed in duplicate on at least three separate occasions. The values are averages based on experiments were measurements differed by less than 15%.

3. Results and discussion

3.1. Hydrogenase–chaperone interactions detected by a two-hybrid assay

Protein–protein interactions between chaperones and apoenzymes are a feature of the biosynthetic pathways of many types of metalloproteins. In the case of [NiFe] hydrogenases, intricate networks of chaperones and accessory factors involved in the early cofactor biosynthesis and insertion steps have been delineated using a non-exported enzyme as a model (e.g. [15,20–22]). Furthermore, Magalon et al. [23] were recently able to detect the specific interaction between a bacterial nitrate reductase subunit and a nitrate reductase-specific chaperone which is required for molybdopterin cofactor insertion using a bacterial two-hybrid system.

Dove et al. [18] described an *E. coli*-based system for measuring protein–protein interactions in vivo. A two-plasmid protocol allows fusion proteins to be constructed separately to the C-terminus of the DNA-binding protein λcI (expressed from plasmid pBT) and to the C-terminus of the α-subunit of RNA polymerase (expressed from plasmid pTRG). Any interaction between fusion partners aligns RNA polymerase correctly onto an engineered test promoter and initiates transcription of a *lacZ* reporter gene [18]. In order to assess the effectiveness of this bacterial two-hybrid system in detecting hydrogenase–chaperone interactions we first tested the system with two proteins that are known to form a complex.

[NiFe] hydrogenase α-subunits are synthesised as precursors with short C-terminal extensions (Fig. 1C). The C-terminal extensions have no role in protein transport but are instead 'assembly peptides' used as staging posts by a number of accessory proteins involved in cofactor insertion [21]. Following successful cofactor loading the C-terminal assembly peptides are cleaved off [21]. The *E. coli* HybG protein has been shown to bind tightly to the precursor form of the catalytic α-subunit of hydrogenase-2 (HybC) during the maturation process [22]. The *hybG* gene was cloned downstream and in frame with the λcI repressor gene on plasmid pBT. Next, the full-length *hybC* gene encoding the precursor form of the hydrogenase-2 α-subunit (preHybC) was cloned into the pTRG plasmid. Following transformation of the KS1 reporter strain with the pBT and pTRG derivatives, increasing amounts of IPTG were used to induce production of the fusion proteins

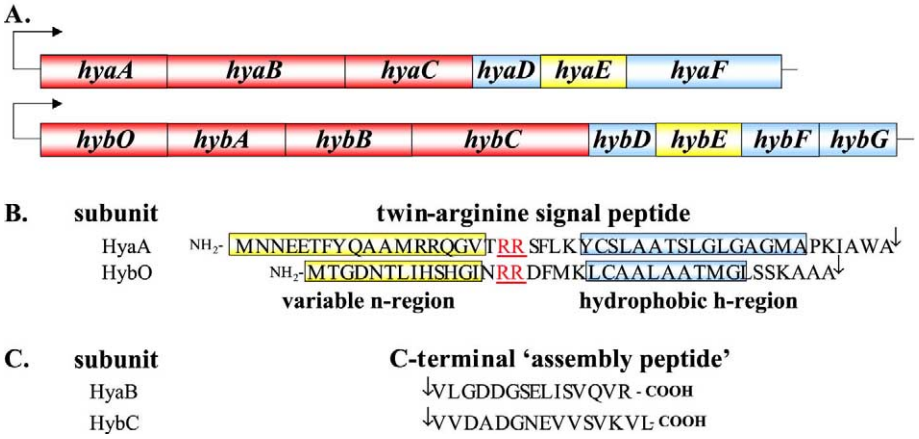

Fig. 1. The uptake hydrogenases of *E. coli*. A: Cartoon representation of the *hya* operon encoding hydrogenase-1 and the *hyb* operon encoding hydrogenase-2. Genes encoding structural subunits of the enzymes are coloured red, genes encoding accessory proteins shown or predicted to be involved in [NiFe] cofactor assembly are coloured blue, and genes of unknown function under investigation in this work are coloured yellow. The direction of transcription is indicated by the arrows. B: Sequence of the twin-arginine signal peptides from the uptake hydrogenase β-subunits. Variable N-terminal domains are highlighted in yellow, hydrophobic sections are highlighted in blue, and the essential twin-arginines are coloured red. Sites of signal peptidase-I cleavage are indicated by the arrows. C: Sequence of the cleavable C-terminal 'assembly peptides' located on the uptake hydrogenase α-subunits. Sites of proteolytic cleavage are indicated by the arrows.

from both plasmids. In the presence of the native pTRG plasmid expression of pBT-HybG lead to the detection of basal levels of β-galactosidase activity at all IPTG concentrations (Fig. 2). However, co-expression of pBT-HybG with pTRG-preHybC resulted in a significant IPTG-dependent increase in LacZ activity confirming the specific interaction between HybG and the hydrogenase-2 α-subunit (Fig. 2). Thus the bacterial two-hybrid system of Dove et al. [18] facilitates the detection of hydrogenase–chaperone interactions.

3.2. HyaE interacts with the HyaA precursor

Next we sought to identify in vivo interactions mediated by the uncharacterised HyaE protein. The *hyaE* gene was cloned downstream and in frame with the λcI repressor gene on plasmid pBT. Next, two forms of the *hyaA* gene encoding the [NiFe] hydrogenase-1 β-subunit were cloned into the pTRG plasmid: the full-length gene encoding the HyaA precursor including N-terminal Tat signal peptide (pTRG-preHyaA), and a truncated gene encoding only the mature form of HyaA minus signal peptide (pTRG-matHyaA). In the presence of the native pTRG plasmid, expression of pBT-HyaE lead to the measurement of only trace levels of β-galactosidase activity at all IPTG concentrations (Fig. 3A). Similarly, expression of the pTRG-preHyaA and pTRG-matHyaA plasmids with native pBT did not activate transcription of the reporter system (data not shown). Thus no non-specific activation of the reporter system was evident in this experiment. Co-expression of pBT-HyaE with pTRG-matHyaA encoding a fusion protein with no twin-arginine signal peptide resulted in only low and unwavering reporter activity (Fig. 3A). However, co-expression of pBT-HyaE with pTRG-preHyaA encoding a fusion protein with an intact Tat signal peptide led to a dramatic IPTG-dependent increase in LacZ activity indicative of a specific interaction between HyaE and the HyaA precursor (Fig. 3A).

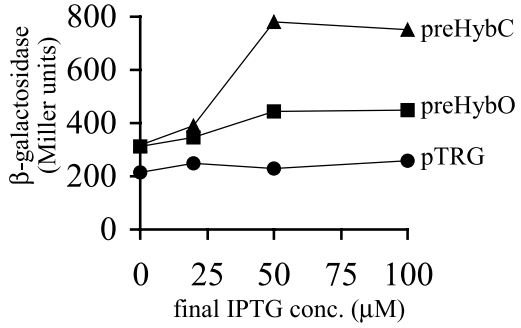

Fig. 2. HybG interacts with the HybC precursor. KS1 cells harbouring pBT-HybG encoding the [NiFe] cofactor chaperone HybG and indicated pTRG derivatives were grown in the presence of IPTG and assayed for β-galactosidase activity. Data marked 'pTRG' are the control experiment with unmodified plasmid, 'preHybC' represents the full-length hydrogenase-2 α-subunit including C-terminal assembly peptide, and 'preHybO' represents the full-length hydrogenase-2 β-subunit including Tat signal peptide. Cells were grown aerobically at 37°C.

To test possible interactions between HyaE and the [NiFe] hydrogenase-2 β-subunit HybO, two forms of the *hybO* gene (encoding full-length precursor and signal-less mature polypeptide) were cloned in frame with the RNA polymerase α-subunit on plasmid pTRG. Again, when the pTRG-preHybO and pTRG-matHybO plasmids were expressed with unmodified pBT no non-specific activation of the reporter system was observed (data not shown). In contrast to the strong interaction recorded between HyaE and preHyaA (Fig. 3A), co-expression of pBT-HyaE with either pTRG-preHybO (containing twin-arginine signal) or pTRG-matHybO (mature form, no signal) revealed only a relatively weak interaction between HyaE and HybO that was not dependent on the presence of a Tat signal peptide (Fig. 3B).

Next, the α-subunits were tested. The *hyaB* gene encoding the catalytic subunit of hydrogenase-1 was cloned into pTRG. Plasmids pTRG-preHyaB and pTRG-preHybC (corresponded to the full-length genes encoding precursor forms of the α-subunits with C-terminal extensions intact (Fig. 1C)) were incapable of activating *lacZ* transcription in isolation (data not shown). Subsequent co-expression of pBT-HyaE with either pTRG-preHyaB or pTRG-preHybC pointed to no strong

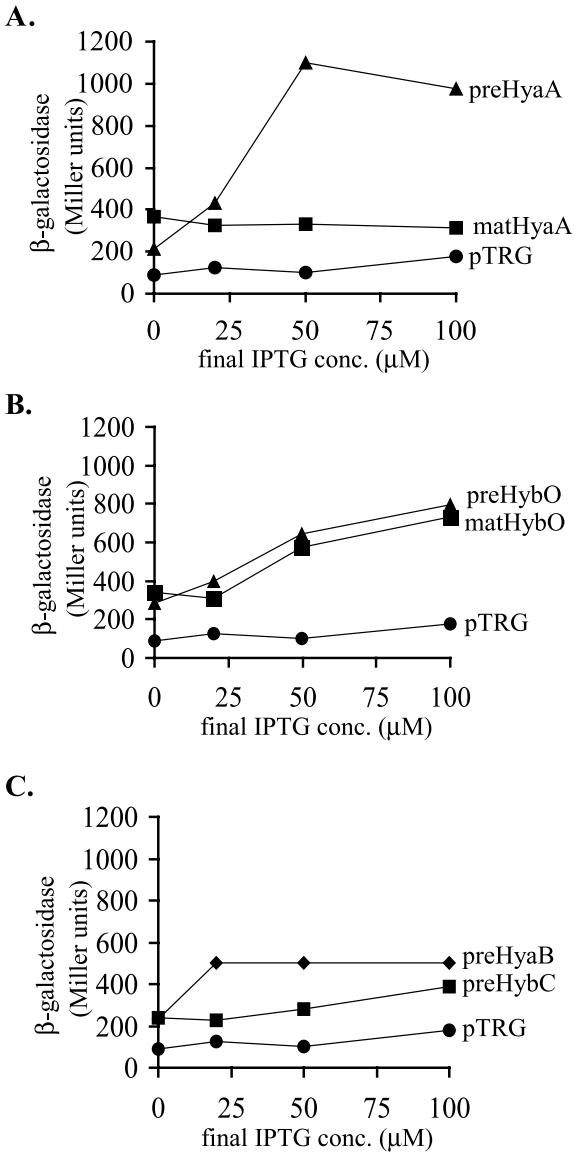

Fig. 3. HyaE interacts with the HyaA precursor. KS1 cells harbouring pBT-HyaE and indicated pTRG derivatives expressing (A) hydrogenase-1 β-subunit in the precursor form with Tat signal peptide ('preHyaA') and the mature form with no signal peptide ('matHyaA'), (B) hydrogenase-2 β-subunit in the precursor ('preHybO') and mature ('matHybO') forms, and (C) uptake hydrogenase α-subunit precursors 'preHyaB' and 'preHybC' (i.e. both containing intact C-terminal assembly peptides), were grown in the presence of IPTG and assayed for β-galactosidase activity. Cells were grown aerobically at 37°C and data marked 'pTRG' are the control experiments with pBT-HyaE co-expressed with unmodified pTRG.

interactions between the HyaE protein and the hydrogenase α-subunits (Fig. 3C).

Taken together, these data point strongly to a role for the HyaE protein in binding the twin-arginine signal peptide-bearing precursor form of the HyaA protein. We therefore classify *E. coli* HyaE as a hydrogenase-1 β-subunit-specific chaperone.

3.3. HybE interacts with the HybO and HybC precursors

The *hybE* gene was cloned downstream and in frame with the λcI repressor gene on plasmid pBT. Expression of pBT-HybE with unmodified pTRG lead to the measurement of very low levels of LacZ activity (Fig. 4A) indicating no non-specific activation of the reporter system by this construct. In this case, however, when pBT-HybE was co-expressed with either the pTRG-preHyaA or pTRG-matHyaA plasmids encoding fusion proteins to precursor and mature HyaA, respectively, no significant interactions could be detected (Fig. 4A).

Co-expression of pBT-HybE with pTRG-preHybO (encoding a fusion to the [NiFe] hydrogenase-2 β-subunit with twin-arginine signal peptide intact) activated transcription from the

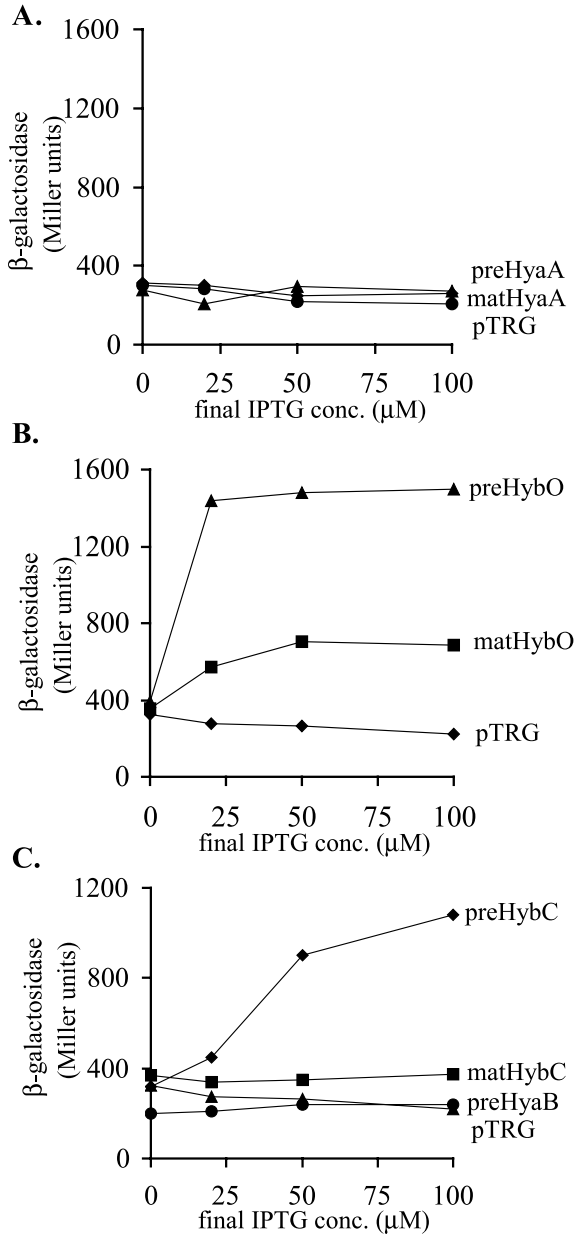

Fig. 4. HybE interacts with the HybO and HybC precursors. KS1 cells harbouring pBT-HybE and indicated pTRG derivatives expressing (A) hydrogenase-1 β-subunit in the precursor, signal peptide-containing form ('preHyaA') and mature, signal-less form ('matHyaA'), (B) hydrogenase-2 β-subunit twin-arginine signal peptide-bearing precursor ('preHybO') and mature ('matHybO') forms, and (C) uptake hydrogenase α-subunit precursors 'preHyaB' and 'preHybC' and the mature form of the hydrogenase-2 α-subunit lacking C-terminal assembly peptide ('matHybC') were grown in the presence of IPTG and assayed for β-galactosidase activity. Data marked 'pTRG' are the control experiments with unmodified plasmid.

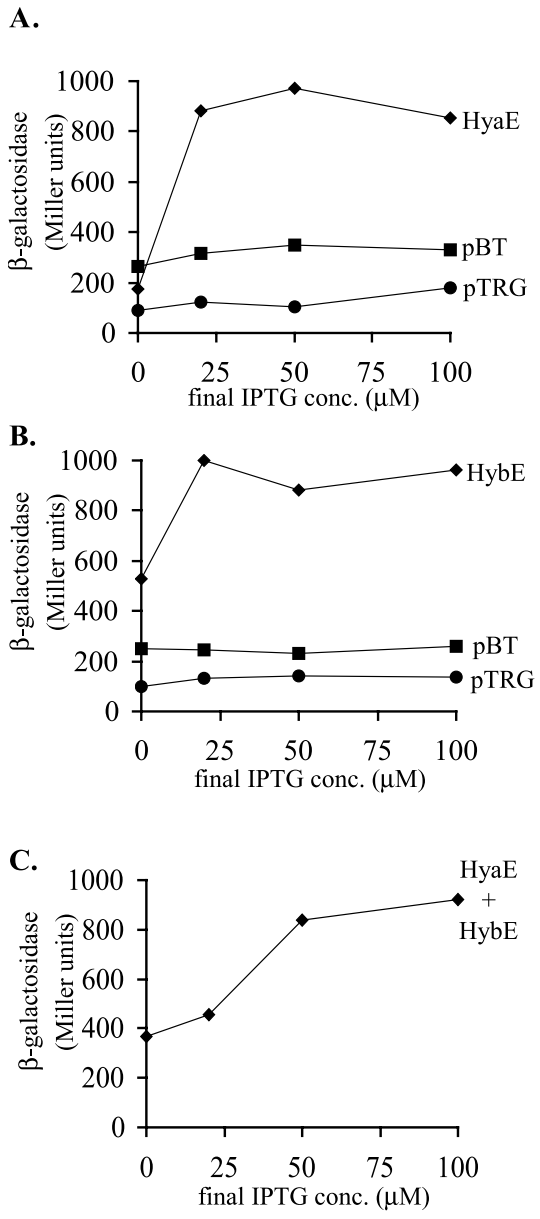

Fig. 5. Intermolecular complexes formed by hydrogenase chaperones. KS1 cells harbouring (A) pBT-HyaE together with unmodified pTRG (control), pTRG-HyaE together with unmodified pBT (control), or pBT-HyaE co-expressed with pTRG-HyaE, (B) pBT-HybE together with unmodified pTRG (control), pTRG-HybE together with unmodified pBT (control), or pBT-HybE together with pTRG-HybE, and (C) pBT-HyaE and pTRG-HybE, were grown aerobically at 37°C in the presence of IPTG and assayed for β-galactosidase activity.

test promoter to a high level (Fig. 4B) indicative of a specific protein–protein interaction. Moreover, HybE was observed to interact with the signal-less mature form of HybO to a much lesser extent (Fig. 4B) pointing to a role for HybE in recognition of a Tat-dependent precursor prior to the protein transport event.

Interestingly, however, the interaction of HybE with the [NiFe] hydrogenase-2 precursor is apparently not restricted to the β-subunit. Co-expression of pBT-HybE with fusion proteins to the α-subunit precursors (with C-terminal extensions intact) showed a clear binding of the hydrogenase-2 α-subunit HybC by the HybE protein (Fig. 4C). Importantly, binding of HybE to HybC was found to be completely dependent on the presence of the 15-residue C-terminal extension to the α-subunit. Co-expression of pBT-HybE with pTRG-matHybO (encoding a truncated HybC fusion with no C-terminal peptide) could not activate transcription from the test promoter (Fig. 4C) indicative of a dramatically weakened interaction.

These data implicate HybE not only in recognition of the Tat signal peptide-bearing precursor form of the [NiFe] hydrogenase-2 β-subunit but also in the binding of the immature form of the signal-less α-subunit. We therefore classify the HybE protein as a hydrogenase-2-specific chaperone.

3.4. Evidence for chaperone cross-talk

To date no biochemical or structural analyses of HyaE or HybE family proteins are currently available. The only twin-arginine signal peptide-binding protein described to date (*E. coli* DmsD [12]) can be inferred by sequence homology to be a dimer [24], or at least 'dimerise' under certain conditions [25]. We therefore exploited our two-hybrid system in an attempt to identify any intermolecular interactions between the HyaE and HybE hydrogenase chaperones.

First, the *hyaE* and *hybE* genes were cloned downstream of, and in frame with, the α-subunit of RNA polymerase on plasmid pTRG. In the presence of the native pBT plasmid, expression of pTRG-HyaE lead to the measurement of only low levels of β-galactosidase activity (Fig. 5A). Co-expression of pBT-HyaE with pTRG-HyaE, however, resulted in a significant IPTG-dependent increase in reporter protein activity (Fig. 5A). These data point strongly to a HyaE–HyaE intermolecular interaction.

Similarly, expression of compatible plasmids harbouring fusions to the *hybE* gene showed that neither plasmid could activate the reporter system in isolation (Fig. 5B). Co-expression of pBT-HybE and pTRG-HybE, however, suggests that HybE is a least a dimer in vivo.

Finally, co-expression of pBT-HyaE with pTRG-HybE demonstrates that there is probably some level of HyaE–HybE intermolecular interaction during hydrogenase assembly (Fig. 5C).

3.5. Concluding remarks

This work is the initial step in the deconvolution of the molecular processes at work in the assembly of Tat-dependent [NiFe] hydrogenases. We have shown that the HyaE protein interacts most strongly with the Tat signal peptide-bearing subunit of hydrogenase-1 HyaA. The HybE protein, on the other hand, interacts specifically with the precursor forms of both α- and β-subunits of hydrogenase-2 and not at all with hydrogenase-1. Since cytoplasmically-oriented [NiFe] hydrogenases bind the same types of cofactors as periplasmic isoenzymes it seems unlikely that HyaE and HybE play a dedicated role in cofactor insertion. It is possible that the chaperones serve either to suppress export of the apoenzymes until maturation is complete, or, in the case of HybE which appears to bind both subunits of the core hydrogenase, prevent premature interactions between the partner subunits. We suggest, therefore, that HyaE and HybE may be involved in 'policing' traffic of these multi-subunit enzymes on the Tat transport pathway.

It is tempting to speculate that HyaE may bind directly to

the twin-arginine signal peptide of HyaA and that HybE may bind to the twin-arginine signal peptide of HybO. This is not inconceivable since the HyaA and HybO signal peptides show obvious sequence differences in the N-terminal domain upstream of the Tat motif that could account for such substrate specificity (Fig. 1B). Indeed, we attempted to address this question directly using our two-hybrid analysis. Unfortunately, the fusion protein constructed between the α-subunit of RNA polymerase and a truncated precursor form of the HybO subunit with no N-terminal domain on the signal peptide proved to be unstable in vivo (data not shown). Clearly, however, our genetic analysis paves the way for future biochemical studies designed to characterise kinetics and specificity of ligand binding to system-specific chaperones required for Tat-dependent hydrogenase assembly.

Interestingly, our data also suggest that HyaE and HybE may work together as part of a larger biosynthetic complex. This is consistent with current models for assembly of complex metalloenzymes such as [NiFe] hydrogenases [21] and molybdopterin-binding enzymes [23] in which interconnected networks of chaperones and assembly factors have been recently described. Some cooperativity of function between HyaE and HybE may also explain why homologues of both chaperones (HoxO and HoxT, respectively) are required for the correct biosynthesis of the solitary Tat-dependent hydrogenase from *R. eutropha* [8].

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