The Extended C-Terminal α -Helix of the HypC Chaperone Restricts Recognition of Large Subunit Precursors by the Hyp-Scaffold Machinery during [NiFe]-Hydrogenase Maturation in *Escherichia coli*

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Abstract

Members of the HypC protein family are chaperone-like proteins that play a central role in the maturation of [NiFe]-hydrogenases (Hyd). *Escherichia coli* has a second copy of HypC, called HybG, and, as a component of the HypDEF maturation scaffold, these proteins help synthesize the NiFe-cofactor and guide the scaffold to its designated hydrogenase large subunit precursor. HypC is required to synthesize active Hyd-1 and Hyd-3, while HybG facilitates Hyd-2 and Hyd-1 synthesis. To identify determinants on HypC that allow it to discriminate against Hyd-2, we made amino acid exchanges in 3 variable regions, termed VR1, VR2, and VR3, of HypC, that make it more similar to HybG. Region VR3 includes a HypC-specific C-terminal α -helical extension, and this proved particularly important in preventing the maturation of Hyd-2 by HypC. Truncation of this extension on HypC increased Hyd-2 activity in the absence of HybG, while retaining maturation of Hyd-3 and Hyd-1. Combining this truncation with amino

acid exchanges in VR1 and VR2 of HypC negatively affected the synthesis of active Hyd-1. The C-terminus of *E. coli* HypC is thus a key determinant in hindering Hyd-2 maturation, while VR1 and VR2 appear more important for Hyd-1 maturation.

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Introduction

Hydrogen (H₂) is an important source of reductant for many bacteria and archaea living in anaerobic environments. The ability to evolve H₂ also allows many fermentatively growing microorganisms to achieve redox balance. The harnessing of these processes, H₂ oxidation and proton reduction, in effective and controlled ways has great potential for alternative energy conversion. Consequently, understanding how [NiFe]-hydrogenases, which catalyze the reversible activation of H₂, are assembled and how they function are important goals of current research. [NiFe]-hydrogenases (Hyd) are found in both archaea and bacteria [Vignais and Billoud, 2007], and the core enzyme is composed of large and small subunits. The catalytically active large subunit harbors the active site

NiFe(CN)₂CO-cofactor, while the small subunit contains iron-sulphur clusters (FeS) that help transfer electrons to and from the bimetallic cofactor, depending on whether the enzyme has a bias towards oxidizing H₂ or proton reduction. In bacteria like *Escherichia coli*, Hyd enzymes are anchored to the membrane by a further subunit, or subunits [Adams and Hall, 1979; Ballantine and Boxer, 1985; Sawers et al., 1985], and these provide a means of electrical contact with the quinone pool and other respiratory complexes [Pinske and Sawers, 2016].

Many anaerobic and facultatively anaerobic archaea and bacteria synthesize more than 1 Hyd, and these enzymes must be made and assembled at appropriate stages of growth or under particular growth conditions. *E. coli* is able to synthesize 3 Hyd enzymes, whereby the first 2 are dedicated to H₂ oxidation while the third produces H₂. Synthesis of these enzymes is controlled on several levels, including transcription, translation, as well as cofactor biosynthesis, enzyme assembly, and membrane targeting [Böck et al., 2006; Penfold et al., 2006], which respond in turn to the physiological status of the cell and whether sufficient resources, such as metal ions and metabolites required for biosynthesis, are available. Although much is understood for several bacteria concerning transcriptional control of the genes and operons encoding Hyd and, to a certain extent, enzyme assembly [Böck et al., 2006; Pinske and Sawers, 2016], there is still much we do not understand about how such processes are controlled and coordinated. In particular, post-translational synthesis, assembly, and insertion of metal cofactors determines when a particular Hyd is active [Lacasse and Zamble, 2016; Pinske and Sawers, 2016; Sargent, 2016], and although in-roads have been made in this research direction, this area is still poorly understood.

Synthesis and insertion of the NiFe(CN)₂CO cofactor into the precursor of the Hyd large subunit is considered a two-step process requiring, minimally, 6 Hyp proteins, which are conserved in all microorganisms that synthesize [NiFe]-Hyd [Jacobi et al., 1992; Jones et al., 2004; Böck et al., 2006]. Some of these proteins have further enzyme-specific copies, like the HypF proteins in *Alcaligenes eutrophus* [Wolf et al., 1998]. *E. coli* has a paralogue of HypC, called HybG, that is encoded within the *hybOABCDEFG* operon [Menon et al., 1994]. HybG has been shown to be required for the maturation of HybC, the large subunit of the Hyd-2 enzyme, which is encoded in the same operon as HybG [Blokesch et al., 2001]. In contrast, the maturation of HycE, the large subunit of the hydrogen-producing Hyd-3, depends solely on HypC [Magalon and Böck, 2000; Blokesch et al., 2004]. More-

over, although HybG is mainly required for Hyd-1 activity, HypC is also capable of maturing HyaB, the large subunit of Hyd-1, in a mutant lacking HybG [Blokesch et al., 2001].

The HypC chaperone was originally identified to interact with the precursor form of HycE, the large subunit of Hyd-3 in *E. coli* [Drapal and Böck, 1998; Magalon and Böck, 2000]. Subsequently, HypC was shown to form a tight complex with HypD [Blokesch et al., 2004] and the HypCD complex, which was crystallized from *Thermococcus kodakarensis*, also associates with HypE and HypF [Bürstel et al., 2012; Watanabe et al., 2012; Stripp et al., 2013; Senger et al., 2017].

The HypDEF proteins are universally required for all 3 Hyd in *E. coli* and, together with the HypC/HybG protein, they perform the first part of the maturation reaction. The iron ion is liganded with 1 CO and 2 CN⁻ groups, and synthesis of this Fe(CN)₂CO moiety is completed on a HypCDEF- or HypDEF-HybG-scaffold complex [Bürstel et al., 2012; Stripp et al., 2013]. This modified group is subsequently inserted into the open active site cavity of the large subunit. There is, meanwhile, strong evidence to indicate that the apo-form of the Hyd large subunit adopts a conformation that facilitates complex recognition and cofactor insertion [Drapal and Böck, 1998; Magalon and Böck, 2000; Jones et al., 2004; Winter et al., 2005].

Members of the HypC-family are small, approximately 10-kDa proteins that share a common OB-β-fold [Wang et al., 2007]. They also have a highly conserved and essential cysteinyl residue at amino acid position 2 on the polypeptide chain [Blokesch et al., 2004]. The N-terminal methionyl residue is removed from the final protein product, and Cys-2 is assumed to coordinate the Fe(CN)₂CO moiety, together with the thiol of the essential Cys-41 (*E. coli* nomenclature) of HypD [Blokesch and Böck, 2006; Bürstel et al., 2012; Soboh et al., 2012; Stripp et al., 2013]. This indicates that HypC is an intermediary between the Hyp-scaffold and the large subunit precursors. Due to the dual coordination of the Fe(CN)₂CO moiety by HypC/HybG and HypD, it is likely that the HypC/HybG proteins transfer the Fe(CN)₂CO to their respective large subunits without completely detaching from HypD, in order to protect the cofactor. It is currently unclear, however, whether HypC or HybG is responsible for cofactor insertion into the large subunit of Hyd-4 in *E. coli* [Pinske and Sawers, 2016].

Nickel is introduced by the HypAB and SlyD proteins after insertion of Fe(CN)₂CO has taken place [Böck et al., 2006; Lacasse and Zamble, 2016]. Apart from nickel-in-

serting HypA and its paralogue HybF, the 2 HypC paralogues are the only Hyp proteins in *E. coli* that are duplicated and that have hydrogenase-specific functions. This indicates that it is these proteins, possibly together with HypD in the Hyp-scaffold complex, which are responsible for guiding the complex to a particular Hyd large subunit precursor and completing Fe(CN)₂CO group insertion. Nevertheless, little is known about how the scaffold complex recognizes individual hydrogenase large subunits, but based on the above considerations, it is conceivable that the HypC and HybG proteins have a key role in discriminating the large subunit precursors.

A recent study identified 3 variable regions in the HypC and HybG proteins that could potentially be involved in the recognition of the Hyd large subunit precursor [Hartwig et al., 2015]. Amino acid residues in these variable regions of HybG that conferred upon it the ability to mature Hyd-3, at least partially, could be identified [Hartwig et al., 2015]. In this study, we have undertaken a detailed analysis of these 3 variable regions in HypC and analyzed their influence on the protein's ability to distinguish between the precursors of the large subunits of Hyd-1 (HyaB), Hyd-2 (HybC), and Hyd-3 (HycE). Our findings demonstrate that the extended C-terminal helix of HypC is decisive in restricting HypC to mature the Hyd-3 and Hyd-1 enzymes.

Results

Competition between Hydrogenase Precursors for HypC and HybG

A strain carrying a deletion in the *hypC* gene no longer synthesized active Hyd-3 but retained active Hyd-1 and Hyd-2 (Fig. 1a). While Hyd-2 activity was comparable to that in the wild-type strain MC4100, Hyd-1 activity was reduced, verifying a role for HypC in Hyd-1 maturation [Blokesch et al., 2001]. Surprisingly, although the introduction of multicopy *hypC* into the *hypC* mutant partially recovered Hyd-3 activity, it abolished detectable Hyd-2 enzyme activity (Fig. 1a). Moreover, while the activity of Hyd-1 was further reduced compared to the *hypC* mutant, use of the more sensitive H₂:nitroblue-tetrazolium (NBT) activity stain [Pinske et al., 2012] nevertheless revealed that residual Hyd-1 activity was still present (Fig. 1b). Introduction of a plasmid encoding HybG^{Strep} into the *hypC* mutant reduced Hyd-3 activity still further (Fig. 1a), but Hyd-1 activity remained at a similar level to that observed when multicopy HypC was introduced into the *hypC* mutant (Fig. 1b). It should be noted that per-

Fig. 1. Multicopy *hypC* out-competes HybG to mature Hyd-3 preferentially. Non-denaturing PAGE analysis of crude extracts (50 µg of protein) derived from the strains MC4100 (Wt), DHP-C ($\Delta hypC$) and DHP-G ($\Delta hybG$) grown in M9 minimal medium with glucose as a carbon source. Strain DHP-C was analyzed with and without pJA1021 (*hypC*) and DHP-G was also analyzed after transformation with pJA1021 or *phybGstrep* (*hybG*). Note that while HypC encoded on pJA1021 carries no tag, HybG carries a N-terminal Strep-tag. After electrophoresis, the gels were stained for Hyd activity with BV-TTC (**a**) or PMS-NBT (**b**) in an atmosphere of 100% H₂ (see Experimental procedures for details). The migration positions of Hyd-1, Hyd-2, and Hyd-3 are shown on the left of the gel.

forming the same experiment with a plasmid encoding HybG without a Strep-tag gave a similar result (data not shown), indicating that the tag was not responsible for the reduction in hydrogenase enzyme activity. These results clearly demonstrated that increasing the gene-dosage of either *hypC* or of *hybG* strongly interfered with the synthesis of the remaining Hyd, presumably through competition for Fe(CN)₂CO. The copy number of pACYC184-based plasmids with a p15A ori is around 10–15 copies per cell, while plasmids with a f1 origin (pASK-IBA) have copy numbers in the range of >100 per cell.

In a reciprocal set of experiments, we examined the effect of deleting the *hybG* gene on the Hyd enzyme profile. A *hybG* allele had no detrimental effect on the activity of Hyd-3, but abolished Hyd-2 activity and strongly reduced Hyd-1 activity (Fig. 1a, b). This finding confirms the role of HybG in the recognition and maturation of HyaB, the catalytic large subunit of Hyd-1 [Blokesch et al., 2001].

Fig. 2. Structural representation of the HypC protein and its orientation in the Hyp-scaffold complex. **a** Alignment of selected HypC family members. HypC_{Ec}, HypC from *E. coli*; HybG_{Ec}, HybG from *E. coli*; HypC_{Tk}, HypC from *T. kodakarensis*; HypC_{Dm}, HypC from *D. mccartyi* strain CBDB1. Amino acid sequence is shown in single-letter code and the variable regions VR1, VR2, and VR3 are highlighted in red, yellow, and green, respectively. **b** Shown is a cartoon view of the NMR solution structure of HypC from *E. coli* [Wang et al., 2007] (PDB 2OT2). The locations of the variable regions VR1, VR2, and VR3 are indicated. All states of the structure are shown simultaneously as ribbons for the VR3 region. The catalytically important Cys-2 has a stick representation, and

an arrow indicates the predicted movement of the α -helix upon binding to HypD. **c** A representation of the structure of the HypC-HypD complex from *T. kodakarensis* [Watanabe et al., 2012] (PDB 3VYR), but with HypC_{Tk} replaced by the *E. coli* solution structure (PDB 2OT2). HypD (a gray surface model) with its [4Fe-4S]-cluster (depicted as spheres), the distance to the bridging, and the catalytic Cys residues (shown as sticks). The concerted binding of the iron moiety is indicated as an Fe-sphere and the locations of the VR1, VR2, and VR3 regions of HypC by colors and arrows (as in **a**; online supplementary Figure S1). **Inset** The same structural model, but with the location of HypE appearing as a light blue surface structure (based on PDB 3VYU).

Introduction of additional copies of the *hypC* gene into the *hybG* mutant did not affect Hyd-3 activity, nor did they restore Hyd-2 activity, but they did augment Hyd-1 activity (Fig. 1). In contrast, introduction of the *hybG* gene restored Hyd-2 enzyme activity to wild-type levels, increasing Hyd-1 activity further, compared to when *hypC* was introduced (Fig. 1b), but reducing Hyd-3 enzyme activity (Fig. 1a). Together, these results suggest that: (1) the HypC and HybG proteins compete for the HypDEF-scaffold complexes; (2) in the absence of HypC, over-production of HybG causes reduction of Hyd-3 and Hyd-2 maturation; and (3) the resulting HypC/HybG-DEF complexes might also compete for hydrogenase large subunit precursors.

HypC Determinants Important for the Recognition of HypE

Alignment of the amino acid sequences of HypC and HybG from *E. coli*, together with the crystallized protein orthologues of HypC from *T. kodakarensis* and the previously described HypC from the *Dehalococcoides mccartyi* strain CBDB1 (Fig. 2a), highlights the 3 variable regions, VR1 (amino acids 13–18 with reference to HypC_{Ec}), VR2 (33–43), and VR3 (75–90); the amino acid range of VR1 and of VR3 has been restricted somewhat compared to the previous definition [Hartwig et al., 2015]. The solution structure of HypC from *E. coli* has been determined [Wang et al., 2007] and the locations of VR1, VR2, and VR3 in the protein are shown in Fig. 2b. However, based

Fig. 3. Influence of amino acid changes in the variable regions of HypC on the Hyd enzyme activity profile. Proteins of crude extracts (50 μ g of protein), derived from the indicated strains after anaerobic growth in M9 minimal medium with glucose as a carbon source, were separated by non-denaturing PAGE (6% w/v polyacrylamide), and subsequently stained for hydrogenase enzyme activity using either BV-TTC (**a**) or PMS-NBT (**b**) (see Experimental Procedures for details). The enzymes responsible for the

respective activity bands are shown on the left of the gel images. The activity-staining band labelled Fdh-O/N signifies the weak H_2 -oxidizing activity associated with the formate dehydrogenases O and N [Soboh et al., 20011] (and acted as a loading control). With the exception of the wild-type MC4100, all other samples were derivatives of strain SHH228 ($\Delta hypC \Delta hybG$) transformed with plasmids (Table 2) encoding the indicated HypC variants.

on the co-crystal structure of the HypC-HypD complex from *T. kodakarensis* [Watanabe et al., 2012], the amino acids in these variable regions are clearly not involved in the interaction with HypD, and they align along an axis on the opposite side to the HypC-HypD interaction surface (Fig. 2c). Therefore, in order to analyze their potential role in HypC recognition of the large-subunit precursors, we decided to make HypC more similar to its paralogue HybG by introducing a series of changes in which different amino acids were added or removed (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000489929). Some of these exchanges were combined to create derivatives with multiple changes in VR1, VR2, and VR3. The changes made in VR1 included the introduction of the HQL tripeptide between amino acids 15 and 16, which is present in HybG but normally absent from HypC, creating HypC (+ HQL). A further amino acid exchange in region VR1 included converting a glycyl residue at position 14 in HypC to an aspartyl residue found in HypC_{Dm} (Fig. 2a), generating

HypC(G14D). Changes introduced in region VR2 included the deletion of the GS dipeptide (positions 34 and 35) to create HypC(-GS) and the GQP tripeptide at positions 40–42 was exchanged for PAD to deliver HypC(PAD). In VR3, 2 C-terminally truncated variants, HypC(Gstop) and HypC(Vstop), which removed 9 and 14 amino acids, respectively, from the C-terminus, were constructed to deliver proteins of a length similar to HybG (82 amino acids) and HypC (71 amino acids) from *D. mccartyi*, respectively.

Genes encoding these variants were introduced into the $\Delta hypC$ - $\Delta hybG$ double null mutant, SHH228 [Hartwig et al., 2015], and after anaerobic fermentative growth in glucose minimal medium, Hyd-containing protein complexes in detergent-solubilized crude extracts derived from these strains were separated in non-denaturing polyacrylamide gel electrophoresis (PAGE), and subsequently stained for H_2 -oxidizing enzyme activity (Fig. 3a). As a control, introduction of the wild-type *hypC* gene restored Hyd-3 and partially restored Hyd-1 activity bands,

Table 1. Formate hydrogenase enzyme activity of *E. coli* SHH228 (*hypC hybG*) transformed with plasmid-encoded HypC amino acid variants

Strain	Relative formate dehydrogenase activity
MC4100	1.63±0.5
SHH228 (<i>hypC hybG</i>)	<0.01
SHH228 + pJA1021 (<i>hypC</i>)	1.07±0.24
SHH228 + phybGstrep	0.08±0.03
SHH228 + pCThypC(+ HQL)	1.42±0.49
SHH228 + pCThypC(– GS)	1.16±0.17
SHH228 + pCThypC(+ HQL/– GS)	1.52±0.54
SHH228 + pCThypC(Vstop)	0.8±0.02
SHH228 + pCThypC(Gstop)	1.13±0.08
SHH228 + pCThypC(+ HQL/– GS/Vstop)	0.14±0.03
SHH228 + pCThypC(+ HQL/– GS/Gstop)	0.51±0.12
SHH228 + pCThypC(PAD)	0.62±0.02
SHH228 + pCThypC(PAD/+ HQL)	<0.01
SHH228 + pCThypC(PAD/+ HQL/Vstop)	<0.01
SHH228 + pCThypC(PAD/+ HQL/Gstop)	<0.01
SHH228 + pCThypC(G14D)	0.7±0.2

Data were obtained from at least 3 independent experiments. Formate hydrogenase complex activity is calculated as nmol H₂ min/mg and expressed as mean ± standard error of the mean.

while introduction of the wild-type *hybG*, encoding C-terminally Strep-tagged HybG, restored weak Hyd-2 and Hyd-1 activities under these conditions (Fig. 3a, b). Removal of the GS dipeptide from VR2 and introduction of the HQL tripeptide into region VR1 in HypC, both individually and combined, essentially did not impair Hyd-3 enzyme activity. This was also reflected in the ability of these mutated genes to restore effective H₂ production to strain SHH228 (Table 1). The combined VR1 and VR2 exchanges did, however, reduce the ability to mature Hyd-1, reflected by a less intense activity band (Fig. 3). The G to D exchange at position 14 in HypC had no effect on in-gel Hyd-3 activity (not shown), but the change reduced H₂ production by approximately 30% (Table 1). Equivalence in the loading of samples was demonstrated by the weak hydrogen-oxidizing activity band due to the formate dehydrogenases Fdh-O and Fdh-N (Fig. 3a) [Soboh et al., 2011].

The C-terminal truncations (VR3 region; online suppl. Fig. S1) also had no strong negative impact on either Hyd-3 enzyme activity of the strain, as revealed by in-gel activity staining (Fig. 3a), or by the measurement of H₂ production (Table 1). However, combining the –

GS and + HQL exchanges with the truncation of the C-terminus reduced H₂ production by approximately 50% when the stop codon was introduced at amino acid position 82 (Gstop). Combining the VR1 and VR2 exchanges with the Vstop truncation (introduction of a stop codon at position 76) reduced H₂ production to approximately 15% of the level observed for strain SHH228 complemented with a plasmid encoding native HypC (Table 1). Hyd-3 enzyme activity was also significantly reduced in the HypC(– GS/+ HQL/Vstop) variant. These combined exchanges also all had a significant negative impact on Hyd-1 maturation (Fig. 3b).

To verify the importance of the amino acid sequence in the loop region VR2 for HypC function, the GQP exchange to PAD (as found in HybG) was analyzed. Hyd-3 activity was partially retained when a plasmid encoding a HypC variant carrying these changes was introduced into SHH228, but H₂ production was reduced by approximately 50% (Fig. 3a; Table 1). Notably, however, Hyd-1 activity was severely impaired and therefore barely detectable when SHH228 was transformed with a plasmid encoding HypC(PAD) (Fig. 3b). Introduction of the HQL amino acids into this construct, and further combining this with truncation of the C-terminus, abolished H₂ production (Table 1) as well as Hyd-3 and Hyd-1 enzyme activities (Fig. 3; data not shown). The effects of these changes indicate that key amino acids in this loop/VR2 region (Fig. 2a) of HypC between β-sheets 3 and 4 are of particular importance for the interaction with HycE and/or for maintaining the stability of HypC (see below).

Analysis of the HypC protein levels after over-production by immunoblotting revealed that while the HypC (Gstop) and HypC(+ HQL/– GS/Gstop) variants could readily be detected, including possible dimeric forms of the proteins as observed previously [Soboh et al., 2013; Albareda et al., 2014], the HypC(PAD/+ HQL/Gstop) variant could not be detected (online suppl. Fig. S2). This suggests that the reason the HypC derivatives with the PAD exchange lacked hydrogenase activity, or showed reduced activity, was because they could not be over-produced in a stable form.

Together, the data indicate that the VR1, VR2, and VR3 regions combined play an important role in Hyd-3 maturation; however, only the combined introduction of the HQL sequence, the removal of GS, and the 14-amino acid truncation of the C-terminus strongly reduced Hyd-3 activity. Exchange of the GQP amino acid sequence for the PAD tripeptide in VR2 severely affected the protein's stability.

Fig. 4. Removal of the extended C-terminal helix of HypC allows it to mature Hyd-2. Proteins of crude extracts (50 µg of protein) derived from the indicated strains after anaerobic growth in M9 minimal medium with glycerol and fumarate as a carbon source (see Experimental Procedures) were separated by non-denaturing PAGE (6% w/v polyacrylamide), and subsequently stained for hydrogenase enzyme activity using BV-TTC. The enzymes responsible for the respective activity bands are shown on the left of the gel image. With the exception of the wild-type MC4100, all other samples were derivatives of strain SHH228 ($\Delta hypC \Delta hybG$) transformed with plasmids (Table 2) encoding the indicated HypC variants.

Truncation of the C-Terminal Helix of HypC Improves HybC Maturation

Use of an alternative anaerobic growth condition of *E. coli* with glycerol and fumarate induces high-level synthesis and activity of Hyd-2, while Hyd-3-dependent H_2 production is strongly reduced, as is Hyd-1 [Sawers et al., 1985]. Analysis of solubilized crude extracts derived from the strains indicated in Fig. 4 revealed that Strep-tagged, native HybG restored some Hyd-2 activity to strain SHH228 ($\Delta hypC \Delta hybG$), while native HypC partially recovered Hyd-1 activity and a trace of Hyd-2, possibly as a consequence of multicopy *hypC*. The weak, anaerobically active Fdh enzymes (labelled Fdh-O/N) acted as loading controls for these experiments. Notably, both HypC variants with C-terminal truncations showed improved Hyd-2 enzyme activity compared with that of native HypC (Fig. 4); Hyd-1 activity was also concomitantly increased. Surprisingly, the HypC(G14D) variant also exhibited a similar increase in Hyd-2 enzyme activity, suggesting this is an important determinant for HybC recognition. Combining the C-terminal truncations with other changes in VR1 and VR2 led to a similar Hyd-2 activity to that observed for plasmid-encoded native HypC, which is in good agreement with the native-looking Hyd-3 and Hyd-1 activity of the former variant in Figure 3. Notably, all hydrogenase activity was abolished when PAD exchanges were combined with changes in VR1 and VR2 (Fig. 4), and this is in accord with an apparent lack of this HypC variant in over-production experiments (online suppl. Fig. S2).

Discussion

Identifying the key amino acid determinants governing the exclusivity of HypC for the large subunit precursor of Hyd-3 of *E. coli* was the focus of this study. Our results, as well as those of a previous study [Magalon and Böck, 2000; Blokesch et al., 2001], confirmed that HypC and HybG compete for the Hyp-scaffold complex, because over-production of either reduces the activity of the corresponding target enzyme of the paralogous chaperone; e.g., the introduction of multicopy *hypC* into a $\Delta hypC$ mutant prevents appearance of Hyd-2 activity (Fig. 1a). It is notable, however, that introducing multicopy *hybG* (up to 100 additional gene copies) into a $\Delta hybG$ mutant reduced Hyd-3 activity, but did not abolish it, while it restored Hyd-2 (and Hyd-1) activity. However, increasing the copy number of *hybG* in a *hypC* mutant by an equivalent amount reduced the Hyd-2 activity. We currently do not fully understand this result, but it suggests that there is a determinant on HybG, that, when the protein is present in multiple copies, either prevents HybG functioning correctly with the Hyp-scaffold complex, or hinders its correct interaction with the hydrogenase large-subunit precursors. Attempts to quantify the levels of HypC and HybG in wild-type cells have so far proved unsuccessful because the lower limit of detection of our antibodies is approximately 10 ng of either purified protein (data not shown). Even loading 50 µg of crude extract from a strain carrying multicopy plasmid *phbGstrep* failed to detect the protein (online suppl. Fig. S3). Assuming, therefore, that HypC or HybG is present at between 0.002 and 0.01%

(1–5 ng in 50 µg of soluble extract) of soluble protein, this equates very roughly to only between 50 and 250 nM of these 10-kDa proteins. This low natural abundance of these proteins might explain why an imbalance in NiFe-cofactor [Pinske and Sawers, 2016] distribution occurs when either HypC or HybG is over-produced.

The data nonetheless suggest that HypC takes precedence over HybG regarding its interaction with the Hyp-DEF-scaffold complex, and/or that HypC interacts more efficiently with its target HycE than does HybG with its principal target HybC. This would also be in accord with the fact that Hyd-3 of the hydrogen-evolving formate hydrogenlyase complex is preferentially made in fermenting *E. coli* cells [Pinske and Sawers, 2016]. Future studies involving comparing these multicopy effects with the influence of mutated genes in single copy, along with quantitative in vitro chemical cross-linking studies coupled with mass spectrometry analysis with purified proteins, will be required to dissect the precise interactions between these chaperones and their client proteins.

Based partially on these observations, it was decided to introduce the HypC variants into a strain lacking both *hypC* and *hybG* to obviate competition between the chaperones, which facilitated interpretation of the effects of the amino acid alterations on hydrogenase maturation; the assumption, based on previous studies [Jacobi et al., 1992; Blokesch et al., 2001], was made between the appearance of hydrogenase enzyme activity and maturation of the corresponding large subunit precursor. This proved an effective strategy and allowed us to demonstrate that a key determinant in preventing HypC from maturing HybC (the Hyd-2 large subunit) was the addition of an approximately 8-amino-acid-long α -helix at its C-terminus [Wang et al., 2007] (Fig. 2b). Truncation of the C-terminus to a length similar to that of HybG, or even to that of the shorter HypC protein from *D. mccartyi*, improved its ability to mature Hyd-2 (see Fig. 4). Importantly, these truncated variants of HypC retained their ability to mature Hyd-1 and Hyd-3. This finding also explains why the short (71 amino acids) HypC from *D. mccartyi*, when heterologously produced in the *E. coli* *hypC-hybG* double-null mutant SSH228, showed promiscuous maturation behaviour in that it was able to mature all 3 *E. coli* enzymes [Hartwig et al., 2015]. A C-terminal extension on the HypC orthologue, HupF, which co-exists with HypC in the cells of the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*, has been shown to stabilize maturation of the target hydrogenase large subunit towards oxygen [Albareda et al., 2012], suggesting that modification or extension of the

C-terminus can add functional specificity to these chaperones.

The other 2 amino acid regions identified as having significant variability between HypC and HybG [Hartwig et al., 2015] are part of the central OB-fold of the protein, whose main function is to facilitate interaction with HypD and generally with the Hyp-scaffold complex [Drupal and Böck, 1998; Wang et al., 2007]. Both regions, VR1 and VR2, are located on loops that align on the same side of the protein as the C-terminus of HypC in the structure of the HypC-HypD complex from *T. kodakarensis* [Watanabe et al., 2012]. Notably, the solution structure of *E. coli* HypC reveals that the C-terminal α -helix has an altered orientation (by approx. 90°) when it is not in complex with HypD (Fig. 2). However, when in complex with HypD, the C-terminus of HypC realigns and appears more constrained within the HypD fold of the *T. kodakarensis* complex (Fig. 2c). A further structure of the HypC-HypD-HypE complex is available [Watanabe et al., 2012] and shows binding of HypE on the “opposite” side of the conserved regions (Fig. 2c, inset). Based on this ternary structure, it is clear that all 3 variable regions of HypC are not involved in the interaction with HypD or HypE, which also is in agreement with the fact that HypE has not been shown to interact directly with HypC [Böck et al., 2006] and would be commensurate with VR1, VR2, and VR3 being available for interaction with the large-subunit precursors.

Both the removal of the GS motif, which is absent in HybG, and the introduction of the HQL tripeptide, which is present in HybG, were remarkably well tolerated by HypC with respect to Hyd-3 (HycE) maturation (based on H₂ production and enzyme activity). Notably, when the GS motif was introduced into HybG and the HQL removed, the HybG variant showed improved Hyd-3 activity and reduced Hyd-2 activity [Hartwig et al., 2015]. These data indicate that regions VR1 and VR2 are also important in distinguishing HycE from HybC. Moreover, altering both of these amino acid motifs in HypC reduced Hyd-1 activity significantly, suggesting that these motifs are important for the recognition of HyaB, the large subunit of Hyd-1; this is also supported by previous findings for HybG [Hartwig et al., 2015]. It is intriguing that, although HybG appears to have a more significant role in maturing HyaB, HypC also seems to be required for maximal Hyd-1 activity during fermentative growth. This might indicate that sub-populations of scaffold complexes containing either HybG or HypC might work together to mature the enzyme, depending on the physiological conditions. It should also be noted that Hyd-1 is a sig-

Table 2. Strains and plasmids

	Genotype	Reference
Strain		
MC4100	F ⁻ , <i>araD139</i> , $\Delta(\arg F-lac)U169$, λ^{-} , <i>rpsL150</i> , <i>relA1</i> , <i>deoC1</i> , <i>flhD5301</i> , $\Delta(fruK-yeiR)725(fruA25)$, <i>rbsR22</i> , $\Delta(fimB-fimE)632(::IS1)$	Casadaban [1976]
DHP-C	MC4100 $\Delta hypC$	Jacobi et al. [1992]
DHB-G	MC4100 $\Delta hybG$	Blokesch et al. [2001]
SHH228	DHP-C $\Delta hybG$	Hartwig et al. [2015]
Plasmid		
phybGstrep	<i>hybG</i> in pASK-IBA3, Amp ^R	Soboh et al. [2014]
pJA1021	pACYC184, <i>hypC</i> ⁺ from <i>E. coli</i> , Cam ^R	Jacobi et al. [1992]
pCThypC(+ HQL)	pJA1021, insertion of HQL after amino acid position 16	our study
pCThypC(-GS)	pJA1021, deletion of GS amino acids at position 34 and 35	our study
pCThypC(+ HQL/- GS)	pCThypC(+ HQL), deletion of GS amino acids at 34 and 35	our study
pCThypC(Vstop)	pJA1021, introduction of a stop codon at position 77	our study
pCThypC(Gstop)	pJA1021, introduction of a stop codon at position 82	our study
pCThypC(+ HQL/- GS/Vstop)	pCThypC(+ HQL/- GS), introduction of a stop codon at position 77	our study
pCThypC(+ HQL/- GS/Gstop)	pCThypC(+ HQL/- GS), introduction of a stop codon at position 82	our study
pCThypC(G14D)	pJA1021, exchange of G for D at amino acid position 14	our study
pCThypC(PAD)	pJA1021, exchange of GQP at amino acid positions 40–43 for PAD	our study
pCThypC(PAD/+ HQL)	pCThypC(PAD), insertion of HQL after amino acid position 16	our study
pCThypC(PAD/+ HQL/Vstop)	pCThypC(PAD/+ HQL), introduction of a stop codon at position 77	our study
pCThypC(PAD/+ HQL/Gstop)	pCThypC(PAD/+ HQL), introduction of a stop codon at position 82	our study

nificantly more abundant enzyme than Hyd-2 during fermentation [Sawers et al., 1985; Sawers and Boxer, 1986].

Only when the truncation of the C-terminal helix in HypC was combined with the changes in VR1 and VR2 could a significant reduction in Hyd-3 activity be observed. This suggests that, as long as the C-terminal extension is present on HypC, HycE can be matured, even if changes are made in VR1 and VR2. The apparent destabilization of the HypC protein by the conversion of the GQP sequence to a PAD sequence (an amino acid sequence found in HybG) at the border of the VR2 region was surprising, indicating that not all changes in this region of HypC are tolerated and that they cause severe structural changes.

We could not identify a specific region or amino acid exchange that improved Hyd-1 maturation relative to Hyd-3 activity; however, as was observed for Hyd-3, most amino acid changes were tolerated, such that some active Hyd-1 enzyme was essentially always made. HypC appears to be the general chaperone for Hyd-3 and, depending on the physiological status of the cell, also for Hyd-1 and this is reflected by the fact that it is encoded within the *hyp* operon in *E. coli* [Jacobi et al., 1992]. The promiscuity shown by HyaB, the Hyd-1 large subunit, towards HypC is also in line with the fact that it does not have its

own specific HypC-family chaperone encoded within the structural gene operon [Menon et al., 1990]. This is in contrast to HybG, which is encoded within the *hyb* operon that also encodes the structural components of Hyd-2 [Menon et al., 1994]. Nevertheless, the fact that HyaB is also matured by HybG indicates that it is flexible with regard to Hyp-scaffold recognition. The aim of future experiments will be to identify the structural determinants on all 3 of the hydrogenase large-subunit precursors that specify HypC/HybG recognition and interaction.

Experimental Procedures

Strains and Growth Conditions

The strains used in this study are listed in Table 2. For routine molecular biology studies, growth was on LB-agar plates or with shaking in LB-broth and at 37 °C [Miller, 1972]. Anaerobic growths were performed at 37 °C as standing liquid cultures. Unless stated otherwise, *E. coli* cells were grown in M9 minimal medium, which included 47.6 mM Na₂HPO₄ × 2 H₂O, 22 mM KH₂PO₄, 8.4 mM NaCl, 20 mM NH₄Cl, 2 mM MgSO₄, 0.1 mM CaCl₂, and 0.1 mM thiamin dichloride [Sambrook et al., 1989]. The growth medium was supplemented with trace element solution SLA [Hormann and Andreesen, 1989]. The carbon source was either glucose (0.8% w/v) or glycerol (0.5% w/v) and sodium fumarate (0.5% w/v) [Sawers et al., 1985]. When required, anhydrotetracycline (AHT) was added at a final concentration of 200 µg/mL and added to cultures

when they had reached an OD_{600 nm} of 0.3. Cells were harvested when cultures had reached an OD_{600 nm} of between 0.8 and 1.2. When required, the antibiotics ampicillin, kanamycin, or chloramphenicol were added to a final concentration of 100, 50, or 12 µg/mL, respectively. Cells were harvested anaerobically by centrifugation at 5,000 g for 15 min and at 4 °C. Cell pellets were used immediately or stored at –20 °C until use.

Plasmid Construction and Site-Directed Mutagenesis

Site-directed mutagenesis of the *hypC* gene was performed using the Quik-Change procedure of Stratagene. The oligonucleotide primers used for mutagenesis are listed in online supplementary Table S1. The authenticity of the mutated *hypC* genes was validated by DNA sequencing. All plasmids were transformed into the appropriate *E. coli* strains as described [Chung et al., 1989].

Preparation of Crude Cell Extracts

E. coli cell paste was re-suspended at a ratio of 1 g wet weight/3 mL in 50 mM MOPS, pH 7, including 5 µg DNase/mL, and 0.2 mM phenylmethylsulfonyl fluoride. Cells were disrupted anaerobically by sonication (30 W power for 5 min with 0.5-s pulses) in a CoyTM chamber under an atmosphere of 95% N₂:5% H₂. Unbroken cells and cell debris were removed by centrifugation for 30 min at 50,000 g and 4 °C. The resultant supernatant was termed the crude extract and, unless otherwise stated, was used for all studies reported herein.

Determination of protein concentration was done as described [Lowry et al., 1951].

Non-Denaturing PAGE, Activity-Staining, and Immunoblotting

Unless otherwise specified, non-denaturing PAGE was performed anaerobically. Separating gels included 0.1% (w/v) Triton X-100 as described [Ballantine and Boxer, 1985]. The crude extracts were incubated with a final concentration of 4% (w/v) Triton X-100 prior to application (usually 50 µg of protein) to the gel, which included 6% (w/v) polyacrylamide. Hydrogenase activity-staining was done in 50 mM MOPS buffer pH 7.0, as described [Sawers et al., 1985], and included 0.5 mM benzyl viologen (BV) and 1 mM 2,3,5-triphenyltetrazolium chloride (TTC). Gels were incubated under an atmosphere of 100% highly pure hydrogen gas. In order to stain specifically for Hyd-1 activity, staining was done in a 100 % hydrogen atmosphere using 0.3 mM phenazine methosulfate (PMS) as mediator and 0.2 mM nitroblue tetrazolium (NBT) as redox dyes [Pinske et al., 2012].

Denaturing PAGE and Immunoblotting

Polypeptides in crude extracts were separated by 12.5 % (w/v) sodium dodecyl sulfate (SDS)-PAGE [Laemmli, 1970] and gels were either stained with Coomassie Brilliant Blue R or transferred to nitrocellulose membranes [Towbin et al., 1979]. For immunodetection, antibodies directed against HypC were used at a dilution of 1:800. The secondary antibody conjugated to horse-radish peroxidase (Bio-Rad, Munich, Germany) was used according to the manufacturer's instructions. Visualization of the antibody-antigen interaction was achieved using the enhanced chemiluminescence reaction (Agilent Technologies).

Measurement of Hydrogen Production

Gas chromatographic determination of hydrogen content in cultures was carried out in Hungate tubes filled with 5 mL of the respective medium and the 10-mL head space was flushed with nitrogen, as described [Pinske et al., 2015]. An aliquot of 200 µL gas phase from the head space was analyzed on a Shimadzu GC-2010 Plus gas chromatograph. Pure nitrogen was used as the carrier gas, and the amount of produced hydrogen was calculated based on a standard curve.

Bioinformatic Analyses

Protein alignment was done using the Muscle algorithm integrated in Jalview [Waterhouse et al., 2009]. Figures of PDB structures were generated using PyMOL (The PyMOL Molecular Graphics System, v1.3, Schrödinger, LLC).

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Disclosure Statement

The authors declare that they have no competing interests.

Author Contributions

C.T., M.W., and K.N. carried out the experiments. C.P. designed and prepared figures. R.G.S. and C.P. drafted the manuscript and conceived the study. All authors read and approved the final manuscript.

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