[NiFe]-hydrogenase maturation *in vitro*: analysis of the roles of the HybG and HypD accessory proteins

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Keywords: Biosynthesis, carbon monoxide, cyanide, nickel, hydrogenase activity, metalloprotein

Summary Statement

Here we demonstrate the *in vitro* reconstitution of active [NiFe]-hydrogenases from inactive intermediates. An anaerobically purified accessory protein complex comprising HybG-HypD and HypE completed maturation of apo-hydrogenases 1 and 2 *in vitro* yielding functional enzymes. The results suggest that a ternary complex carrying an intact $Fe(CN)_2CO$ moiety is required for reconstitution of enzyme activity.

short (page heading) title
In vitro maturation of [NiFe]-hydrogenase

Summary

[NiFe]-hydrogenases (Hyd) bind a nickel-iron-based cofactor. The Fe ion of the cofactor is bound by two cyanide ligands and a single carbon monoxide ligand. Minimally six accessory proteins (HypA through HypF) are necessary for $NiFe(CN)_2CO$ cofactor biosynthesis in *E. coli*. It has been shown that the anaerobically purified HypC-HypD-HypE scaffold complex carries the $Fe(CN)_2CO$ moiety of this cofactor. Here, we have purified the HybG-HypDE complex and used it to successfully reconstitute *in vitro* active Hyd from *Escherichia coli*. HybG is a homologue of HypC that is specifically required for the maturation of Hyd-2 and also functions in the maturation of Hyd-1 of *Escherichia coli*. Maturation of active Hyd-1 and Hyd-2 could be demonstrated in extracts derived from HybG- and HypD-deficient *E. coli* strains by adding anaerobically purified HybG-HypDE complex. *In vitro* maturation was dependent on ATP, carbamoyl phosphate, nickel, and reducing conditions. Hydrogenase maturation was prevented when the purified HybG-HypDE complex employed in the maturation assay lacked a bound $Fe(CN)_2CO$ moiety. These findings demonstrate that it is possible to isolate incompletely processed intermediates on the maturation pathway and to use these to activate apo-forms of [NiFe]-hydrogenase large subunits.

Introduction

Hydrogenases catalyse the reversible oxidation of dihydrogen [1] and are of industrial interest because it is hoped to adapt their active site to develop robust catalysts for H_2 production [2, 3, 4]. [NiFe]-hydrogenases (Hyd) are of particular interest because some of them function in the presence of O_2 [5]. Hyd are uniformly composed of a large subunit harbouring a bimetallic $NiFe(CN)_2CO$-center and a small subunit harbouring one to three iron sulphur clusters. Additional subunits may be present to transfer the electrons, e.g. to pyridine nucleotides or to anchor the Hyd to the membrane [6]. The nickel ion of the bimetallic active site is coordinated via four highly conserved cysteinyl thiolates [7], while two of the thiolates additionally bind the Fe ion. The Fe ion carries one CO and two CN^- ligands and these are proposed to maintain a low-spin Fe(II) state [8, 9].

Maturation of the large subunit requires the coordinated activities of at least six Hyp accessory proteins, which catalyse the synthesis of the diatomic ligands, their attachment to the Fe ion and the subsequent insertion of the nickel ion [10, 11]. The HypC, D, E, and F proteins together synthesize the $Fe(CN)_2CO$ moiety [10, 11]. A deletion in any of the genes encoding the Hyp proteins results in a defective hydrogen metabolism phenotype [10]. The HypA and HypB proteins are specifically involved in acquisition and insertion of the nickel ion and their requirement can be obviated by supplementation of the growth medium with high concentrations of nickel [10]. The proposed roles of the individual Hyp accessory proteins in the maturation of Hyd are summarized in the current model of Hyd large subunit presented in Fig. 1. Synthesis of the cyano ligands is initiated by HypF, which catalyzes the ATP-dependent transfer of the carbmoyl moiety of carbamoylphosphate (CP) to the C-terminal cysteinyl of HypE [12, 13]. In a further ATP-dependent reaction the HypF-HypE heterotetrameric complex dehydrates the thiocarboxamide to thiocyanate [14]. The cyano group is then transferred to a Fe ion bound by the HypC-HypD protein complex [15, 16]. The source of the CO ligand has not been definitively resolved. Exogenously provided CO_2 can be excluded as a precursor of the CO ligand [17]; however recent finding suggests that metabolically generated CO_2 is a possible precursor of CO [18, 19, 20]. HypD has a low-potential [4Fe-4S] cluster and is therefore likely to be the only Hyp protein capable of performing redox chemistry and

presumably delivers electrons for CO generation, as well as for the transfer of the CN^- groups to the Fe ion [21]. As the HypC chaperone also interacts specifically with the precursor form of the large subunit [22] it likely delivers the $Fe(CN)_2CO$ centre to the apo-large subunit. Nickel is inserted by the combined actions of the HypA, HypB (a GTPase) and the prolyl *cis/ trans* isomerase SlyD but only after insertion of the Fe centre has been completed [23]. The inserted nickel ion acts as a recognition template for a hydrogenase-specific endoprotease, which cleaves the polypeptide chain three residues C-terminal to the fourth cysteine that coordinates the $NiFe(CN)_2CO$ active center [24]. It is assumed that this proteolytic step results in a conformational change causing active site closure and completion of maturation of the large subunit.

The genome of *E. coli* encodes four Hyd but only three of these have been characterised functionally [25, 26]. Many microorganisms synthesize more than one Hyd and often orthologues of certain Hyp proteins are present in these organisms [1,10]. For example, in *E. coli* a second HypC orthologue, HybG, and a second HypA orthologue, termed HybF, exist [27, 28]. These orthologues are necessary to mature a particular hydrogenase large subunit, probably by acting as a substrate-specific adaptor facilitating interaction of a particular hydrogenase large subunit with its cognate set of maturation proteins. HybG and HybF are essential for HybC (Hyd-2) maturation [28, 29].

Most, if not all, proteins involved in the biosynthesis and assembly of the $NiFe(CN)_2CO$-cofactor have been identified, but much concerning the molecular details of metallocluster synthesis remains to be elucidated. To fully understand how the $NiFe(CN)_2CO$ active site is synthesized and to identify the precise sequence of events, an approach is required in which the complete biosynthetic pathway can be reconstructed *in vitro* using only purified proteins. Such an *in vitro* system will allow elucidation of individual steps in biosynthesis of the active site and will facilitate future manipulation of cofactor biosynthesis for catalytic optimization of the enzymes [30-35]. In this study, we show that an anaerobically purified and functional $HybG_{strep}$-HypDE complex can be used to reconstitute *in vitro* active Hyd-1 and Hyd-2 from immature intermediates.

Materials and methods

Strains, plasmids, and growth conditions

All *E. coli* strains and plasmids used in this study are listed in Table 1. For overproduction of proteins, *E. coli* strains were transformed with the appropriate plasmid and grown in modified TB medium [19], containing 100 µg/ml of ampicillin or 50 µg/ml kanamycin at 37 °C on a rotary shaker until an optical density of 0.4 at 600 nm was reached. Gene expression was induced by addition of 0.3 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) followed by incubation at 30 °C for 3-5 h. Cells were harvested (OD_{600nm} of 1.0) by centrifugation for 30 min at 5000 *g* at 4 °C. Cell pellets were used either immediately or stored at -20 °C until use. Crude extracts used for *in vitro* maturation of hydrogenase were prepared from *E. coli* cells cultivated anaerobically at 30 °C in buffered TYEP medium supplemented with 0.8% (w/v) glucose.

Strain construction

Strains were generally constructed by introduction of mutations from *E. coli* donor strains into recipient strains of MC4100 by P1*kc*-mediated phage transduction according to [38] (see Table 1).

Plasmid construction

Chromosomal DNA from MC4100 was used as the template for amplification of the *hypD, E, F* and *hybG* genes via PCR. The resulting fragments were digested with NdeI and HindIII and ligated into NdeI-/HindIII-digested pET28A to generate the plasmids phypD, phypE and phypF, which encoded a fusion protein with an N-terminal His-tag. The *hybG* gene was ligated into NdeI-/HindIII-digested pET30 plasmid. The resulting phybG plasmid encoded C-terminally His-tagged HybG. To generate pT-hypDEFhybGstrep, pT-hypDEFCStrep [15] was digested with NdeI and BamH1 releasing the *hypC* gene. The PCR-amplified *hybG* gene was subsequently digested with BsaI and ligated into the BsaI restriction site of pASK-IBA3 (IBA, Göttingen, Germany), to generate pASK-hybG encoding C-terminally Strep-tagged $HybG_{Strep}$. The *hybG* gene from pASK-hybG was then ligated into the NdeI and BamH1 restriction site of pT-hypDEF

Preparation of Crude Extracts and Protein Purification.

All steps were carried out under anaerobic conditions in an anaerobic chamber (Coy Laboratories, Grass Lake, USA) and at 4 °C unless stated otherwise. Wet cell paste from BL21(DE3) transformed cells containing Strep-tagged HybG-HypDE, Strep-tagged HypC-HypD complex or Strep-tagged HybG was resuspended at a ratio of 1:3 (w/v) in buffer W (100 mM Tris-HCl and 150 mM NaCl, pH 8.0) including 2 mM sodium dithionite, 5 mg/L DNase, and 0.2 mM PMSF. Cells were disrupted by sonication (40 W for 10 min with 0.5 s pulses). Unbroken cells and debris were removed by centrifugation for 30 min at 5000 *g* at 4 °C, and the supernatant is henceforth referred to as the crude extract. The crude extract derived from 5 to 10 g wet weight of cells was used for anaerobic purification of proteins. Proteins were isolated by chromatography on a StrepTactin-Sepharose column (5 mL; IBA, Goettingen, Germany) using gravity-flow. Unbound proteins were removed by washing the column with five column volumes of buffer W. Recombinant protein was eluted with buffer W including 5 mM desthiobiotin. Desthiobiotin was subsequently removed by passage through a series of Hi-Prep PD10 desalting columns (GE Healthcare) equilibrated with buffer W and connected to an ÄKTA apparatus (GE Healthcare). Proteins were eluted and concentrated by centrifugation at 5000 *g* using centrifugal filters (Amicon Ultra, 50 K, Millipore, Eschborn, Germany). His-tagged-HypF, -HypE, -HypD and -HybG protein variants were purified from BL21(DE3) transformed with the appropriate plasmid derivative (Table 1). Wet cell paste was resuspended at a ratio of 1:4 (w/v) in buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, and 2 mM sodium dithionite) including, 5 mg/L DNase, and 0.2 mM PMSF. Crude extracts derived from approximately 5 g of wet cell paste were loaded onto a 1.5 × 10 cm column of Co^{2+}-charged resin (Talon resin, Clontech). The column, which had been previously equilibrated with buffer A was washed with five column volumes of the same buffer followed by three column volumes of buffer A supplemented with 10 mM imidazole and then three column volumes of buffer A supplemented with 20 mM imidazole to remove non-specifically bound proteins. His-tagged proteins were subsequently eluted with buffer A containing 300 mM imidazole. Imidazole was removed by desalting as described above.

***In vitro* hydrogenase maturation reactions**

The complete *in vitro* maturation reaction mixture contained 50 µl (10 g/l) of extract derived from the corresponding deletion strain as indicated, varying concentrations of purified $HybG_{Strep}$-HypDE complex, 2.5 mM ATP, 50 µM carbamoyl phosphate, 2 mM sodium dithionite (DTH) , 50 µM $FeSO_4$, 100 µM $NiCl_2$ in 50 mM MOPS-buffer (pH 7.0). Reactions were carried out in a total volume of 100 µl inside 2 ml stoppered vials for 30 min at 25 °C under a nitrogen atmosphere and subsequently placed on ice. When aerobic incubation of samples is indicated, the substrate and protein

Biochemical Journal Immediate Publication. Published on 03 Sep 2014 as manuscript BJ20140485

mixture was mixed and incubated for 30 min in the presence of air prior to activity measurement. When purified His-tagged HypD, HybG, HypE, and/ or HypF proteins were included in the reaction mixture, proteins were mixed in an equimolar ratio (2 µM of each protein per reconstitution reaction). When desalted extracts were used in the reaction assay, the cell extracts were desalted anaerobically using a Hi-Prep PD10 desalting column equilibrated with MOPS buffer, pH 7 prior to use in the reaction.

FT-IR spectroscopy

Steady-state Fourier-transform infrared (FT-IR) spectra were recorded on a Tensor27 spectrometer (Bruker Optik, Ettlingen, Germany) equipped with a silicon crystal attenuated total reflection (ATR) cell of two active 60° reflections (Smith Detection, Warrington, USA) as described earlier [20]. All experiments were performed in the absence of oxygen under a 99:1 N_2/ H_2 protective atmosphere. A drop of 1 µL sample solution was dried on top of the crystal surface to judge the amount of protein and integrity of the active site CO and CN contributions probed by the evanescent wave. For each spectrum, 5000 scans were averaged to enhance the S/N ratio. Samples were probed under dry N_2 and low frequency water contributions from 2400 – 1800 cm^{-1} were subtracted by spline function. Band positions fit best to unbiased Gaussian bell curves. All spectra are normalized to amide II band heights.

Non-heme Fe determination

Iron content was determined by inductively coupled plasma mass spectrometry (ICP-MS) as described earlier [41]. For ICP-MS analysis 0.1 mg of purified HybG-HypDE complex was used and samples were analyzed for Fe, Ni, Zn, and Cu.

Polyacrylamide gel electrophoresis

Purified proteins (10 to 20 µg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 15% (w/v) polyacrylamide. Non-denaturating PAGE was performed using 7.5% (w/v) polyacrylamide gels, pH 8.5 and included 0.1% (w/v) Triton X-100 in the gels. Samples (20 to 250 µg of protein) were incubated with 4% (w/v) Triton X-100 prior to application to the gels. The functionality of maturation assay was tested on the basis of hydrogenase activity under aerobic condition at RT after non-denaturing PAGE was performed as described in [42], except that the buffer used was 50 mM MOPS pH 7.0.

Other methods

Determination of protein concentration was performed as described in [43] with bovine serum albumin (BSA) as standard. Hydrogenase enzyme activity was determined according to [42] except that the buffer used was 50 mM MOPS, pH 7.0. Activities were measured using 1 cm path-length anaerobic cuvettes using an Uvicon 900 dual-wavelength spectrophotometer. One milli-unit of activity (mU) corresponds to oxidation of 1 nmol of hydrogen gas per min. Experiments were performed at least twice and each experiment was performed in duplicate. The values reported are the standard error for one experiment. While some variation is absolute activity was observed between biological replicates, the effects noted in the text below were always the same and highly reproducible. This is reason why the data from a representative experiment are presented.

Results

Analysis of purified-HybG$_{Strep}$-HypDE complex

Recent studies have demonstrated that purified, Strep-tagged HypCD complex from strain BL21/pT-hypCDEF also carries the $Fe(CN)_2CO$ moiety [19, 44]. In the present study a plasmid pT-hybG-hypDEF was constructed by replacing *hypC* in pT-hypCDEF with the homologous gene *hybG* from *E. coli*, which delivered a protein

Biochemical Journal Immediate Publication. Published on 03 Sep 2014 as manuscript BJ20140485

carrying a *C*-terminal Strep-tag. The accessory proteins $HybG_{Strep}$, HypD, HypE and HypF required for [NiFe]-hydrogenase maturation were overproduced in BL21(DE3). $HybG_{Strep}$ was isolated by anaerobic affinity chromatography from crude extracts clearly and SDS-PAGE analysis of the protein revealed it formed a ternary complex with HypD and HypE (henceforth $HybG_{Strep}$-HypDE complex). As shown in Fig. 2A, the complex was close to homogenous; however, the amount of HypE in the complex was variable and generally sub-stoichiometric. After concentration the complex was deep brown in colour consistent with the presence of an iron-sulphur cluster and non-heme iron [19]. Metal analysis of anaerobically isolated $HybG_{Strep}$–HypDE complex determined by ICP-MS showed that the as-isolated complex contained 4.4 ± 0.27 mol Fe per one mol of ternary complex. Molar ratios was calculated based on the molecular masses of HybG-HypDE trimer. Notably, the amount of Ni, Cu, Co, and Zn was below the detectable limit. Analysis of aerobically isolated complex identified 3.2 ± 0.29 Fe mol per mol protein. Notably, the amount of Ni, Cu, Co, and Zn was below the detectable limit. Analysis of aerobically isolated complex identified 4.0 ± 0.3 Fe mol per mol protein. These findings strongly suggest that the additional Fe, apart from that in the [4Fe4S] cluster of HypD, is labile and was lost during aerobic purification. Based on the results of earlier studies [19], it is possible that the additional Fe ion(s) on $HybG_{Strep}$–HypDE is required for the coordination of the diatomic ligands. Analysis of the as-isolated $HybG_{Strep}$-HypDE complex by FT-IR spectroscopy revealed strong absorption bands at 2096 cm^{-1}, 2073 cm^{-1}, and 1962 cm^{-1} (Fig 2B), which correspond to the vibrational frequencies of Fe(II)-CN^- and Fe(II)-CO, respectively. A similar spectroscopic fingerprint was found for the HypC-HypD-HypE complex (Fig. 2B) [19, 44] and has been attributed to the $Fe(CN)_2CO$ cofactor [45]. An additional band centred at 2337 cm^{-1} can be assigned to the asymmetrical stretch vibration of CO_2, which has been previously identified in the HypC superfamily [18]. Together, these findings indicate that the $HybG_{Strep}$-HypDE complex functions as an acceptor for the Fe-bound CN^- and CO ligands.

The $Fe(CN)_2CO$-containing $HybG_{Strep}$-HypDE complex can complete Hyd-1 and Hyd-2 maturation *in vitro* yielding active enzymes

Figure 3 shows an activity stain specific for hydrogen-oxidizing hydrogenases performed after separation of protein complexes in a crude extract derived from anaerobically grown wild type *E. coli* by native polyacrylamide gel electrophoresis (native-PAGE). Under the conditions tested, four activity bands can be detected in extracts derived from the wild type MC4100 (see lane 1). The fastest migrating species corresponds to Hyd-1, while the three more slowly migrating species are due to Hyd-2 [46]. Extracts derived from mutants deficient in any one of the Hyp accessory proteins required for hydrogenase maturation failed to reveal any activity band (e.g. see lanes 6 and 7 in Fig. 3). Addition of anaerobically purified $HybG_{Strep}$-HypDE complex to an anaerobically prepared *in vitro* reaction mixture containing an extract of an *E. coli* strain carrying a lesion in *hypD* (DHP-D) or *hybG* (DHP-G) restored the activities of both Hyd-1 and Hyd-2 (Fig. 3, lanes 3 and 8). It should be noted that the amount of crude extract derived from MC4100 (wild type) used in the experiment was 7-fold lower than that used in the experiments shown in lanes 2 though 8. No hydrogenase activity band was detected when a crude extract derived from a *ΔhypE* or *ΔhypF* deletion strain (DHP-E and DHP-F) was used in the reaction assay, even if purified $HybG_{Strep}$-HypDE complex was included (lanes 6 and 7). Aerobic pre-incubation of the reaction shown in lane 3 revealed that oxygen prevented reconstitution of activity (lane 2). Aerobic incubation of the crude extract derived from MC4100 for the same period of time had no effect on activity of Hyd-1

or Hyd-2 (data not shown), which is also consistent with the results of early studies in which both enzymes could be purified in the presence of oxygen [42, 25] Reconstitution of active Hyd-1 and Hyd-2 critically depended on the presence of ATP, carbamoyl phosphate, $NiCl_2$, and the reductant dithionite (DTH), henceforth referred to as activation mixture (AM). In the absence of the AM hydrogenase activity could not be restored, as shown in lane 5 of Fig. 3.

The specific hydrogenase enzyme activities of the various reconstitution reactions were determined and are presented in Table 2. These reactions were performed with as-isolated crude extract or after desalting of the crude extract to remove small molecular weight components (see Methods). Because HybG can be used for the maturation of Hyd-1 and Hyd-2, but not for Hyd-3 [10], total hydrogenase activity of a crude extract derived from DHJ725 (a Δ*hycE* mutant that lacks Hyd-3 but has fully functional Hyd-1 and Hyd-2) was determined and served as a positive control and represents the maximal activity that could be reconstituted (Table 2). The anaerobic reconstitution reaction including the $HybG_{Strep}$-HypDE complex together with the complete activation mixture with crude extract yielded an activity that was approximately 11 % of the activity of the DHJ725 extract (reaction 3 in Table 2). This represented the sum of the activities of Hyd-1 and Hyd-2. When untreated crude extract was replaced by desalted extracts the activity recovered was only 7.5% of that in strain DHJ725. No hydrogenase activity could be measured in a reaction performed with desalted crude extract of the *hypD* mutant but lacking $HybG_{Strep}$-HypDE in the reaction (reaction 4). A similar result was observed for the untreated crude extract but the background activity was slightly higher (Table 2). These results demonstrate that the $HybG_{Strep}$-HypDE complex is essential for reconstitution of hydrogenase enzyme activity. Omission of ATP, CP, $NiCl_2$ or DTH significantly reduced the activity obtained when a desalted cell extract from the *hypD* mutant was used (reactions 5-7 in Table 2). Omission of $NiCl_2$ or $FeSO_4$ from the activation mixture reduced the hydrogenase activity by approximately 65% and 40%, respectively (reactions 7 and 9 in Table 2). The total hydrogenase activity obtained when an untreated crude extract was used was generally significantly higher (Table 2), suggesting that low-molecular weight components might be delivered by the cytoplasmic fraction. Even when the untreated crude extract was used, the presence of reductant was essential to reconstitute maximal hydrogenase activity (reaction 8 in Table 2). No hydrogenase activity could be reconstituted under aerobic conditions or when the $HybG_{Strep}$-HypDE complex had been treated with EDTA prior to incubation in the reconstitution reaction (reaction 10, 11 in Table 2). Moreover, after aerobic treatment, then subsequent anaerobic incubation, no hydrogenase activity could be recovered indicating that oxygen irreversibly inhibited the reconstitution reaction. Together, these findings suggest that oxygen inactivated one or more components of the reaction and that the chelator inhibits the activity of the $HybG_{Strep}$-HypDE complex.

The presence of the $Fe(CN)_2CO$ moiety on the purified complex is suggested to be necessary for the reconstitution, as employing a $HybG_{Strep}$-HypDE complex, isolated from a Δ*hypF*-mutant strain (DHPF-2) could not restore hydrogenase activity (Table 2 compare reaction 12 and 13). FT-IR and metal analyses have demonstrated that purified HypCDE complex does not harbour the $Fe(CN)_2CO$ moiety if it is purified from Δ*hypF* background [44].

We hypothesized that the $Fe(CN)_2CO$ moiety would accumulate on the HybG-HypDE complex in a mutant strain FTD147, which lacks the large subunits of Hyd-1 through 3. Surprisingly, the complex isolated from FTD147 was very labile and did not lead to recovery of hydrogenase activity in *vitro* (Table 2, reaction 14). This suggests that the

Biochemical Journal Immediate Publication. Published on 03 Sep 2014 as manuscript BJ20140485

apo-large subunit might be required to stabilize the HybG-HypDE complex *in vivo*. Together, these results strongly suggest that a $HybG_{Strep}$-HypDE complex carrying the $Fe(CN)_2CO$ moiety and isolated from an *E. coli* strain with apo-large subunits is required to allow reconstitution of hydrogenase activity and suggest that the complex delivers the Fe-center that serves as a precursor of the [NiFe]-active center.

Kinetics of $HybG_{Strep}$-HypDE complex-dependent *in vitro* hydrogenase reconstitution

The $Fe(CN)_2CO$-containing $HybG_{Strep}$-HypDE complex, when mixed with a crude extract derived from a *hypD* mutant, can complete biosynthesis of Hyd-1 and Hyd-2 yielding active enzymes. Figure 4a shows the increase of hydrogen-oxidizing activity as a function of $HybG_{Strep}$-HypDE concentration. The saturation profile of the reaction suggests that $HybG_{Strep}$-HypDE is not the limiting component in the reconstitution reaction. Adding more than 0.5 µM $HybG_{Strep}$-HypDE complex to the reaction mixture did not significantly increase hydrogenase activity, suggesting that the activation reaction is limited by an additional factor (or factors) in the crude extract. The time course shown in Fig 4b revealed that reconstitution of hydrogenase enzyme activity was complete within 10 min when the reaction was performed at room temperature. No significant increase or decrease of activity after 120 min incubation was observed.

Addition of purified HypE and HypF improve the *in vitro* reconstitution

To determine whether a component of the crude extract was limiting in the reconstitution reaction a defined concentration of purified $HybG_{Strep}$-HypDE complex was used and increasing amounts of a crude extract derived from strain DHP-D (Δ*hypD*) was added. The results shown in Fig.4c demonstrate that hydrogenase activity increased with increasing amounts of crude extract, confirming that a component delivered in the crude extract was limiting in the reconstitution reaction. The standard reconstitution reaction has 0.5 mg of total protein. By increasing this to 2 mg it was possible to almost double the hydrogenase activity attained. Performing the same reaction with an extract derived from strain DHP-F2 (Δ*hypF*) and then adding back purified His-tagged HypFE revealed that the limiting component was was HypF or possibly HypE, which together form a functional complex [13, 14]. Addition of purified HypF alone to the reconstitution reaction that included $HybG_{Strep}$-HypDE complex, desalted extract of DHP-D (Δ*hypD*) and AM had no effect on the level of hydrogenase activity obtained (Table 3). However, addition of both purified HypF and HypE increased the hydrogenase activity two-fold (reaction 3 and 4 in Table 3). No hydrogenase activity could be measured when HypD or HybG individually, or in combination after separate purification, was used in the reconstitution assay instead of the $HybG_{Strep}$-HypDE complex (reactions 5, 6, and 7, respectively). This result indicates that the ternary complex carrying the $Fe(CN)_2CO$ cofactor is essential for successful reconstitution of enzyme activity. Anaerobically purified $HypC_{Strep}$-HypD-HypE complex charged with the $Fe(CN)_2CO$ moiety (see Figure 2B) could not replace the function of $HybG_{Strep}$-HypDE in reconstitution of hydrogenase activity (Table 3 reaction 8). This result indicates that the specificity for HybG in the activation of the Hyd-1 and Hyd-2 precursors was retained *in vitro* [27]. When the extract derived from DHP-D was replaced with an extract from the *hypF* mutant DHP-F2, addition of $HybG_{Strep}$-HypDE complex plus HypF reconstituted only 35% of the activity measured when the DHP-D extract was used (compare reactions 3 with 11 in Table 3). However, addition of purified HypF and HypE increased the activity to 80% compared to the same reaction in which the DHP-D extract was used (compare reactions 4 with 12 in Table 3). Finally, when an extract prepared from the

hypE mutant DHP-E was incubated with the $HybG_{Strep}$-HypD complex plus purified HypE and HypF (reaction 16) roughly 26% of the activity measured with the DHP-D extract could be measured. $HybG_{Strep}$-HypD complex alone or HypE protein alone was unable to restore hydrogenase activity to extracts of the DHP-E strain (reaction 14, 15). Together, these data suggest that an equimolar ratio of HypE and HypF is needed for reconstitution of hydrogenase enzyme activity and if either component is missing from an extract the counterpart component is either inactive or degraded.

Discussion

In this work we report on a reconstitution system for the *in vitro* activation of [NiFe]-hydrogenases 1 and 2 of *E. coli*. Previous studies had demonstrated that it is possible to reconstitute functionally active enzyme *in vitro* using biochemical complementation of two crude extracts derived from mutants both lacking active hydrogenase [40]. In this study, we have been able to isolate anaerobically a ternary complex of the accessory proteins Hyb-HypD and HypE that restores hydrogenase activity to an extract containing only apo-forms of the large subunits of Hyd-1 and Hyd-2. The *in vitro* $NiFe(CN)_2CO$ assembly and maturation system is still nevertheless sub-optimal, because it only recovered approximately 10% of the hydrogen-oxidizing activity that was present in a crude extract of a strain harbouring fully active Hyd-1 and Hyd-2. This finding indicates that at least one further component, or an activity, necessary for the efficient maturation of these enzymes is limiting. This was confirmed when it was noted that increasing the amount of crude extract in the reconstitution assay resulted in increased hydrogenase activity. This limitation could be partially compensated by adding anaerobically purified HypE and HypF proteins, suggesting that the activity of these components might be one reason for the low recovery of activity.

In vitro maturation of hydrogenase activity is oxygen-sensitive and critically relies on the presence of an electron source

A strong dependence on ATP, CP, nickel and dithionite as reductant could be demonstrated. Omission of any of these components significantly decreased the restored hydrogenase activity when desalted cell extracts were used in the reconstitution assay. The absence of an iron source had less impact on *in vitro* hydrogenase reconstitution, suggesting that sufficient Fe ions were present in the assay mixture. Indeed, FT-IR analysis of the $HybG_{Strep}$-HypDE complex confirmed the presence of a bound $Fe(CN)_2CO$ moiety, whose signatures at 1962, 2073, and 2095 cm^{-1} indicate coordination of the three diatomic ligands to an Fe ion. Oxygen caused irreversible inactivation of the reconstitution mixture, while the effect of a lack of reductant was reversible. Oxygen clearly inactivates one or more components in the system and this could be due to instability of the $Fe(CN)_2CO$ moiety toward oxygen [19]. Although Hyd-2 is described as an oxygen-sensitive enzyme, it is possible to maintain activity in crude extracts in the presence of oxygen for the duration of the reconstitution assay [42], strongly supporting the contention that a component of the HybG-HypDE ternary complex is oxygen-sensitive; Hyd-1 is in any case an oxygen-tolerant enzyme [25, 42, 47]. This proposal is also in agreement with the fact that the chelator EDTA impedes the reconstitution reaction, suggesting that the $Fe(CN)_2CO$ moiety represents the labile component.

An electron source is required for the transfer of the CN^- groups to Fe [15] and minimally two further electrons would be required for the generation of CO if CO_2 is the substrate [48]. It is therefore likely that dithionite serves as a source of reductant

in the *in vitro* assay, possibly replacing the role of the physiological electron donor to HypD [15, 20].
The HybF (homolog of HypA), HypB together with SlyD accessory proteins are specifically involved in acquisition and insertion of the nickel ions subsequent to insertion of the $Fe(CN)_2CO$ group into the apo-hydrogenase large subunit [28, 49, 23]. Presumably, because a comparatively high concentration of nickel ions was included in the reconstitution reaction the requirement for over-production of these metallo-chaperones *in vitro* was obviated, which is in agreement with previous findings [24, 50]. Even in the absence of additional nickel the activity of the reaction mix including desalted cell extracts was 40 % of that of the positive control (reaction 7 in Tab. 2), suggesting that some nickel ions in bound form are delivered by extract.

HypC(HybG)-HypDE forms the central complex in $Fe(CN)_2CO$ biosynthesis
It has been proposed that the $Fe(CN)_2CO$ moiety of the [NiFe]-cofactor is sequentially assembled on the HypCD(HypE) complex as a molecular scaffold [15, 19, 44]. A similar function can now be suggested for HybG-HypDE, which is required for cofactor insertion into Hyd-2 and Hyd-1 in *E. coli* [51]. Isolation of Strep-tagged HybG resulted in co-purification of HypD and near-stoichiometric amounts of HypE. In our previous studies on the HypCD complex, low but detectable amounts of HypE were co-purified as part of the complex [19]. The current findings suggest that HypE forms a more stable interaction with HybG-HypD than with the HypC-HypD complex. As with the HypCD complex our biochemical and FT-IR analyses indicate that HybG-HypDE also assembles and likely delivers the $Fe(CN)_2CO$ moiety to its destination substrates apo-HyaB and apo-HybC. It was only possible to restore hydrogenase activity when the anaerobically purified HybG-HypDE complex was used in the reconstitution assay together with the activation mixture and a crude extract derived from either DHP-D (Δ*hypD*) or DHP-G (Δ*hybG*). It was not possible under otherwise identical conditions to obtain hydrogenase activity when the individually purified HybG and HypD proteins were employed. This finding indicates that the physical complex of the three proteins is essential to accomplish effective maturation. Hydrogenase activity also could not be restored when extracts of DHP-E (Δ*hypE*) and DHP-F2 (Δ*hypF*) mutants were used in combination with anaerobically purified HybG-HypDE complex. Activity could, however, be detected when in addition to HybG-HypDE complex anaerobically purified HypF and HypE were added to the mixture. HypF and HypE are required for CN^- synthesis but these findings might indicate that they are also important for efficient transfer of $Fe(CN)_2CO$ moiety from HybG-HypDE complex to the large subunit of Hyd-1 and Hyd-2 (e.g. facilitating substrate channelling). Notably, despite only HypF being absent from a DHP-F2 extract, addition of purified HypF on its own failed to recover hydrogenase activity. A similar observation was made when purified HypE together with the HybG-HypDE complex were added to an extract of DHP-E. Together, our data suggest that the presence of the $Fe(CN)_2CO$ moiety on HybG-HypDE is necessary for the reconstitution of hydrogenase activity *in vitro*, perhaps signifying a biochemically active ternary complex. No hydrogenase activity could be detected under any reaction conditions when “uncharged” HybG-HypDE complex was employed in the maturation assay. Future studies will focus on demonstrating that the HybG-HypDE complex transfers the $Fe(CN)_2CO$ moiety to the apo-large subunit of Hyd-1 and Hyd-2.
The historical use of *in vitro* reconstitution systems using purified components has been crucial to gain insight into the underlying biochemistry of metalloprotein maturation [52]. For example, a detailed functional understanding of the nitrogenase

accessory proteins and synthesis of the iron-molybdenum cofactor [32, 35] and the recent major advances in understanding of [FeFe]-hydrogenase active site biosynthesis only were achieved using *in vitro* reconstitution systems [30, 33, 53]. Despite the structural differences between [NiFe]- and [FeFe]-hydrogenases and nitrogenase, it is apparent that their biosynthetic pathways share some common features, including the involvement of molecular scaffolds and metallocluster carrier proteins [54]. Increasing our understanding how complex metallo-cofactors are biochemically assembled also has the potential to inspire the design of catalysts for the industrial production of H_2 and other commodities [55].

Acknowledgements
The authors are indebted to Gary Sawers for discussion and support. The work conducted in the authors' laboratories was supported by the region Saxony-Anhalt. STS gratefully acknowledges the Focus Area Nanoscale of the Freie Universität Berlin for financial support and Joachim Heberle for on-going support and by the BMBF (H2 design cells) to J.H.

Author contribution
Basem Soboh designed the research; Basem Soboh, Sven T. Stripp , Ute Lindenstrauss Claudia Granich, Mahwish Javed, Martin Herzberg and Claudia Bielak performed the research; Basem Soboh and Sven T. Stripp analysed the data and interpreted the results of the experiments; and Basem Soboh wrote the paper

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Table 1 Strains and plasmids used in this study

Strains/plasmids	**Genotype**	**References**
MC4100	F$^-$ *araD139 α(argF-lac)U169 ptsF25 deoC1 relA1 flbB5301 rspL150*$^-$	[36]
DHJ725	MC4100 *ΔhycE*	[37]
BL21(DE3)	F-ompT hsdSB(rB-,mB-)gal dcm (DE3)	Novagen, USA
FTD147	MC4100 *ΔhyaB ΔhybC ΔhycE*	[38]
DHP-D	MC4100 *ΔhypD*	[39]
DHP-E	MC4100 *ΔhypE*	[39]
DHP-F2	MC4100 *ΔhypF*	(12)
DHP-G	MC4100 *ΔhybG*	This study
Plasmids		
pT-hypCDEF	pT7-7,hypD, HypE, hypCstrep, hypF, Amp^R	(15)
pT-hybG-hypDEF	pT7-7,hypD, HypE, hybGstrep, hypF, Amp^R	This study
phypF	pET28A, hypF, km^R	[40]
phypE	pET28A, hypE, km^R	[40]
phypD	pET28A, hypD, km^R	[40]
phybGHis	pET30, hybG, Amp^R	This study
phybGstrep	pASK-IBA3,hybG,Amp^R	This study

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Table 2 Requirements for HybG-HypDE complex-dependent *in vitro* reconstitution and maturation of [Ni-Fe] hydrogenase

Reaction No.	[a] Reaction mixture supplement	Hydrogenase activity (mU/ml)	
		Crude extracts	Desalted extracts
1	Only extracts of DHJ725	774 ± 124	702 ± 87
2	[b] Complete,extracts of DHJ725	714 ± 95	696 ± 81
3	[c] Complete$^{BI21(DE3)}$	81 ± 17	51.9 ± 8
4	Minus GDE	6.3 ± 1.5	0.95 ± 0.2
5	Minus ATP	50.7 ± 6.6	14.7 ± 4
6	Minus carbamoyl phosphate	43.8 ± 5.7	12.3 ± 3.1
7	Minus $NiCl_2$	79.6 ± 19	21.2 ± 6.5
8	Minus DTH	28.4 ± 9.6	11.3 ± 3.5
9	Minus $FeSO_4$	63.7 ± 8.5	33.0 ± 15
10	[d] Complete^{aerobic}	10.5 ± 2.8	3.1 ± 0.9
11	[e] Complete^{EDTA}	10.9 ± 3.4	8.2 ± 1
12	[f] Complete^{WT}	48.4± 5.8	41.5± 7.3
13	[g] Complete$^{\Delta hypF}$	2.8 ± 1.7	2.8 ± 1.7
14	[h] Complete$^{\Delta Apo\text{-}Hyd}$	6.2 ± 1.9	2.8 ± 1.8

[a] The complete *in vitro* maturation assay can be found in Materials and Methodes. Experiments were performed at least twice and each experiment was performed in duplicate. The values reported are the standard error for one representative experiment.

[b] Total hydrogenase activity of a crude extracts derived from DHJ725 (a Δ*hycE* mutant that lacks Hyd-3 but has fully functional Hyd-1 and Hyd-2) was determined and served as a positive control representing the maximal activity that could be reconstituted.

[c] HybG-HypDE complex employed in the maturation assay was isolated from BL21(DE3)

[d] Complete reaction mixture was incubated for 30 min in the presence of air prior to activity measurement.

[e] HybG-HypDE was desalted after treating with 10 mM EDTA prior to incubation in the reaction.

[f] HybG-HypDE employed in the maturation assay was isolated from wild type strain MC4100

[g] HybG-HypDE has no $Fe(CN)_2CO$ moiety, was isolated from a ΔhypF-mutant strain (DHPF-2).

[h] HybG-HypDE was isolated from a mutant strain FTD147, which lacks the large subunits of three hydrogenases

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Table 3. *In vitro* complementation of hydrogenase using various extracts of hydrogenase defective strains and the effect of addition purified Hyp-proteins

Reaction No.	[a] Reaction mixture	Hydrogenase activity (mU/ml)
1	Only DHP-D	6.4 ± 1.5
2	DHP-D + GDE	83.5 ± 25
3	DHP-D + GDE + F	88 ± 10
4	DHP-D + GDE + F + E	192 ± 14
5	DHP-D + D	5.7 ± 1.5
6	DHP-D + G	7.3 ± 2.4
7	DHP-D + D + G	3.26 ± 1
8	DHP-D + CD	4.5 ± 1.3
9	Only DHP-F	4.9 ± 0.6
10	DHP-F+ GDE	29.5 ± 6.5
11	DHP-F GDE + F	31.2 ± 6.2
12	DHP-F + GDE + F+ E	153 ± 17
13	Only DHP-E	6.7 ± 0.6
14	DHP-E + GDE	5.6 ± 0.6
15	DHP-E + GDE + E	4.5 ± 0.8
16	DHP-E + GDE + F + E	50 ± 11

a)The *in vitro* maturation reaction mixture contains 0,5 mg of extracts derived from the corresponding deletion strain as indicated plus activation mixture of (2.5 mM ATP, 50 µM carbamoyl phosphate, 2 mM sodium dithionite, 50 µM $FeSO_4$, 100 µM $NiCl_2$ in 50 mM MOPS-buffer pH 7) When purified proteins [HybG-HypDE (GDE); HypF (F); HypE (E); HypD (D); HybG (G) or HypCD (CD)] were included in the reaction mixture as indicated, proteins were mixed in an equimolar ratio of 2 µM of each protein per reconstitution reaction. Reactions were carried out in a total volume of 100 µl inside 2 ml stoppered vials for 30 min at 25 °C under a nitrogen atmosphere. Experiments were performed at least twice and each experiment was performed in duplicate. The values reported are the standard error for one representative experiment.

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Figure legends

Figure-1. Current model for NiFe(CN)$_2$CO biosynthesis and insertion into the hydrogenase large subunit precursor. ***(I)*** The HypF–HypE complex catalyzes the ATP-dependent transfer of the carbamoyl moiety from carbamoylphosphate to HypE to generate the thiocarboximide, which is followed by dehydration to thiocyanate by HypE. ***(II)*** The HypC protein family (HypC or HybG) delivers Fe and CO_2 to HypD, where CO is generated and the cyano groups are transferred to the iron by HypEF. ***(III)*** The HypCDE complex delivers the Fe(CN)$_2$CO group to the precursor of the hydrogenase large subunit.

Figure-2. Analysis of purified HybG$_{Strep}$-HypDE complex. **(A)** SDS-PAGE of purified HybG$_{Strep}$-HypDE complex. Lane 1 purified HybG$_{Strep}$-HypDE complex (6 µg) was separated by SDS-PAGE (15% w/v polyacrylamide) and stained with Coomassie Brilliant Blue. Lane M, PageRuler-Plus prestained molecular mass marker (Fermentas) in kDa. **(B)** FT-IR analysis of purified HybG$_{Strep}$-HypDE complex. Samples were probed on a three-reflection silicon ATR crystal in the absence of O_2. Spectra showing purified HypCD and HybG-HypDE carrying the Fe(CN)$_2$CO moiety of the NiFe(CN)$_2$CO cofactor, as well as CO_2 which has been identified in HypC and HybG superfamily.

Figure- 3. *In vitro* hydrogenase maturation reactions analysed by native PAGE. The functionality of Hyd-1 and Hyd-2 maturation assay was analyzed after separation of proteins in the reaction mixes by native-PAGE followed by staining for Hyd enzyme activity at RT under aerobic condition. Lane 1, positive control of 20 µg crude extract from MC4100 (wild type); lane 2, reaction performed under aerobic conditions and included 140 µg crude extract from strain DHP-D, 50 µg of HybG$_{Strep}$-HypDE complex plus complete activation mixture; lane 3, as lane 2 but the reaction was performed under strictly anaerobic conditions; lane 4, as lane 3 but only 10 µg of HybG$_{Strep}$-HypDE complex was added; lane 5, as lane 3 but lacking activation mixture; lane 6, as lane 3 but a crude extract derived from DHP-F2 was used; lane 7, as lane 3 but a crude extract derived from a DHP-E was used; lane 8, as in lane 3 but a crude extract derived from a DHP-G mutant was used.

Figure- 4 HybG-HypDE complex-dependent *in vitro* reconstitution and maturation of [Ni-Fe] hydrogenase. Hydrogen-uptake activity was determined by following the reduction of benzyl viologen at 578 nm. *In vitro* reconstitution and maturation assay was performed exactly as described in the Methods. **(A)** Titration of hydrogenase maturation assay with HybG-HypDE complex. The indicated concentrations of ternary complex were used. **(B)** Effect of preincubation on the *in vitro* hydrogenase maturation. The reaction mixture was scaled up for 25X reaction, aliquots of 50 µl were taken at the indicated times and were frozen in liquid N_2 until activity determination. **(C)** Dependence of *in vitro* maturation activity of hydrogenase on extract concentration of DHP-D (●) or DHP-F (Δ). When DHP-F extracts was used, purified HypE and HypF were added in an equimolar ratio at a concentration of 2µM to the reconstitution assay that included HybG-HypDE complex

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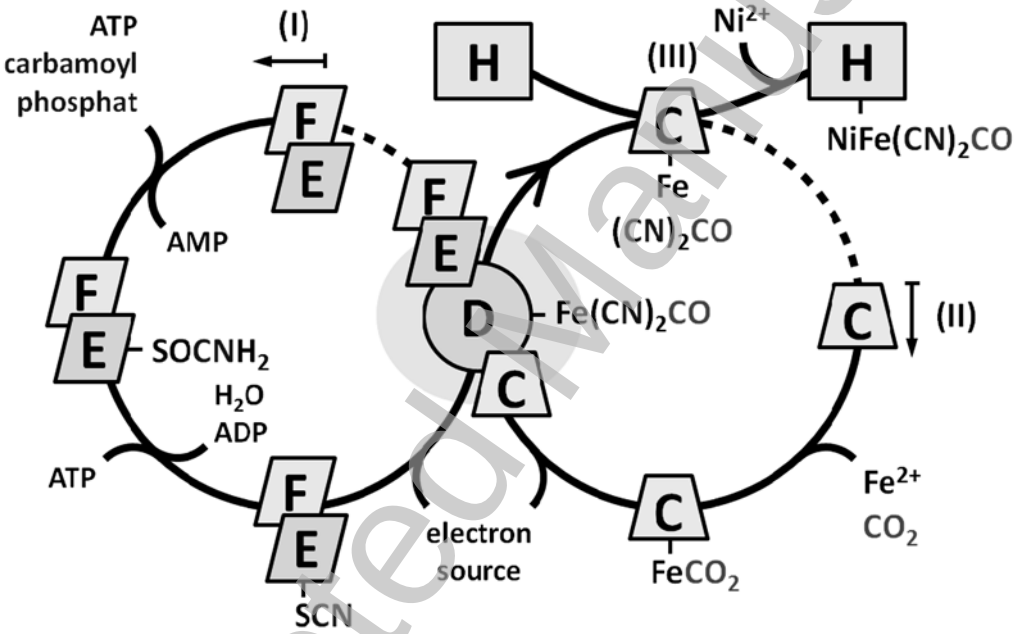

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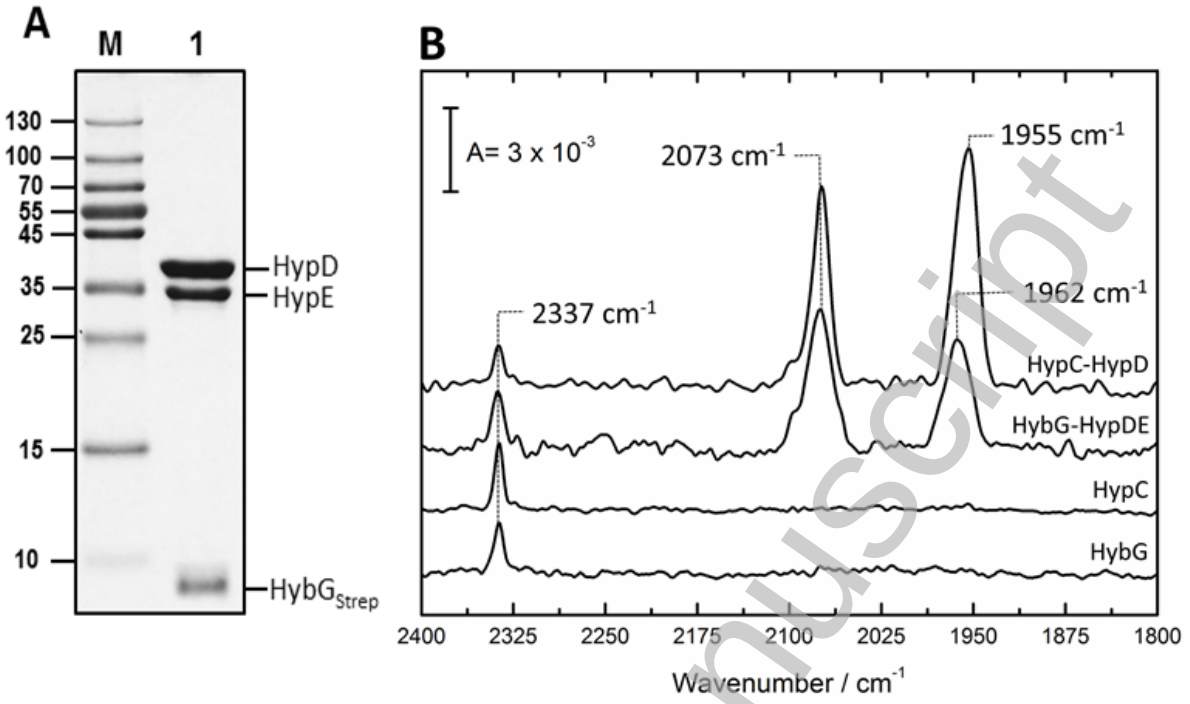

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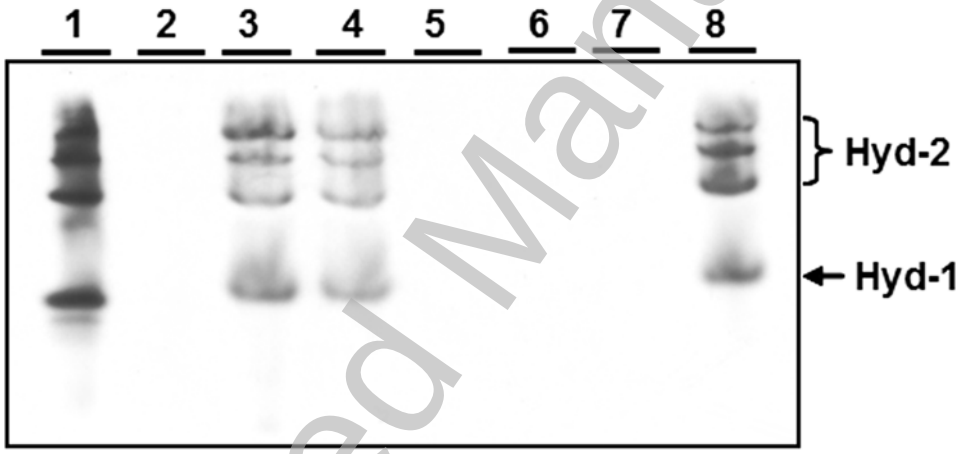

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