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A model system for [NiFe] hydrogenase maturation studies: Purification of an active site-containing hydrogenase large subunit without small subunit

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Abstract The large subunit HoxC of the H_2-sensing [NiFe] hydrogenase from *Ralstonia eutropha* was purified without its small subunit. Two forms of HoxC were identified. Both forms contained iron but only substoichiometric amounts of nickel. One form was a homodimer of HoxC whereas the second also contained the Ni–Fe site maturation proteins HypC and HypB. Despite the presence of the Ni–Fe active site in some of the proteins, both forms, which lack the Fe–S clusters normally present in hydrogenases, cannot activate hydrogen. The incomplete insertion of nickel into the Ni–Fe site provides direct evidence that Fe precedes Ni in the course of metal center assembly.

Keywords: [NiFe] hydrogenase; Metal center assembly; Maturation; *Ralstonia eutropha*

1. Introduction

Hydrogenases, enzymes that catalyze the interconversion of hydrogen into protons and electrons, are widespread among microorganisms [1,2]. The facultative chemolithoautotrophic β-proteobacterium *Ralstonia eutropha* H16 hosts three [NiFe] hydrogenases. The membrane-bound hydrogenases (MBH) and the cytoplasmic soluble hydrogenase (SH) facilitate energy conservation. The regulatory hydrogenase (RH) belongs to a subgroup of H_2-sensing hydrogenases. It initiates a signal transduction chain regulating MBH and SH gene expression [3]. The RH is comprised of a large subunit, HoxC, harboring the Ni–Fe site and a small subunit, HoxB, containing Fe–S clusters. In contrast to standard dimeric [NiFe] hydrogenases from, e.g., *Desulfovibrio gigas* [4], the RH forms a $(HoxBC)_2$ double dimer complexed with a tetramer of a histidine protein kinase [5]. Like standard [NiFe] hydrogenases, the RH contains a $Ni–Fe(CO)(CN)_2$ cofactor [6]. Spectroscopic investigations of wildtype and mutant RH proteins, however, have revealed unusual features of both its Ni–Fe site and its Fe–S clusters [7,8]. Most notably, the RH is insensitive to inhibition by dioxygen and its function does not require reductive reactivation.

Structural information from the Ni–Fe site of RH is expected to shed light on the mechanism of aerobic H_2-sensing. The use of Fe-specific spectroscopic techniques for the analysis of the active site iron is hampered by the presence of Fe-S clusters in the small subunit. To establish a model system for studying the Ni–Fe site in the absence of other Fe containing cofactors, we have constructed an expression plasmid carrying the structural gene of the RH large subunit, *hoxC*, without genetic information for the RH small subunit, HoxB. This construct enabled us to purify for the first time a [NiFe] hydrogenase large subunit without its corresponding small subunit. Biochemical properties of the HoxC derivative are described in this study, and spectroscopic features of its Ni–Fe site are inspected in the accompanying study [9].

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli JM109 [10] was used for cloning procedures, and *E. coli* S17-1 [11] was used for conjugative plasmid transfers between *E. coli* and *R. eutropha*. A *Strep*-tag II-encoding 39-bp sequence was fused to the 5′ end of *hoxC* by inverse PCR using the artificial primers 5′-ATGgctagctggagccacccgcagttcgaaaaaggcgccATGGAAC-GTTTGGTGGTGGGGCCGTTCAACCGCGTTGAGGGC-3′ and 5′-CACCACCAAgctagccatgctcgcctcCTATTTCAGCCGCGTCTTG-CGAATGGCTGGC-3′ (lowercase denotes bases that differ from the original sequence) and plasmid pCH594 [12] as the template. The amplification product was cut with *Nhe*I and religated producing plasmid pCH1044. Subsequently, the 360-bp *Sac*II fragment from pCH594 was replaced by the modified 399-bp *Sac*II fragment from pCH1044 yielding pCH1045. To generate a 459-bp in-frame deletion in *hoxB*, the 3318- and 593-bp fragments obtained after a total *Acc*65I digest of pCH1045 were ligated to give pCH1027. Subsequently, the 1449-bp *Pml*I/*Spe*I fragment of pCH1045 was cloned into the 3749-bp *Pml*I/*Spe*I fragment of pCH1027 to yield pCH1028. Finally, the 2.4-kb *Hin*dIII/*Spe*I fragment of pCH1028 was cloned into the broad-host range vector pEDY309 [12] to give pGE537. Plasmid

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Abbreviations: RH, H_2-sensing regulatory hydrogenase; SH, soluble hydrogenase; MBH, membrane-bound hydrogenase; AAS, atomic absorption spectroscopy; TRXF, total reflection X-ray fluorescence; PAGE, polyacrylamide gel electrophoresis; TCEP, Tris(2-carboxyethyl)- phosphine; LB, Luria broth; FGN, fructose-glycerol minimal medium; CV, column volume; V_e, elution volume; V_0, void volume; BSA, bovine serum albumine; ADH, alcohol dehydrogenase

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pGE377 containing the entire *hoxBC* genes was used as a control [12]. The plasmids were conjugatively transferred to *R. eutropha* HF574 [13], a derivative of *R. eutropha* H16 (ATCC 17699; DSM 428).

2.2. Media and growth conditions

E. coli strains were grown in Luria broth (LB). *R. eutropha* strains were grown in modified LB medium containing 0.25% (w/v) sodium chloride or in mineral salts medium [14] containing 0.4% fructose or a mixture of fructose and glycerol (0.2% (w/v) each; fructose-glycerol minimal medium (FGN)) as carbon sources. Solid media contained 1.5% (w/v) agar. Antibiotics were: 15 μg tetracycline mL^{-1} for *R. eutropha*, and 15 μg tetracycline mL^{-1} or 100 μg ampicillin mL^{-1} for *E. coli*.

2.3. Purification of $HoxC_{ST}$

Strep-tagged HoxC ($HoxC_{ST}$) was purified from *R. eutropha* HF574(pGE537). The cells were grown in FGN medium at an 8 l scale (sixteen 2-l flasks each containing 500 ml medium) and harvested by centrifugation. The cell pellet was resuspended in buffer A (100 mM Tris–HCl, pH 8.0, 150 mM NaCl; 1 ml buffer per 1 g of cells), and passed twice through a French pressure cell at 1100 psi. Cell debris was removed by centrifugation for 45 min at 90000 × *g* at 4 °C. Using a Biocad Sprint Workstation (Applied Biosystems), the soluble extract was applied to a *Strep*-Tactin Superflow column (2 ml bed volume, IBA, Göttingen, Germany). The column was washed with 10 column volumes (CV) of buffer A. Proteins were eluted with 6 CV of buffer A containing 5 mM desthiobiotin. Protein-containing fractions were pooled and concentrated by ultrafiltration using an Amicon Ultra-15 (Millipore) device to obtain a volume less than two milliliters. The sample was applied to a gel filtration column (HiLoad 16/60 Superdex 200 prep grade, Amersham Pharmacia) and isocratically eluted using 10 mM Tris–HCl, pH 8.0. Fractions were analyzed by Coomassie-stained SDS–PAGE. Homogenous $HoxC_{ST}$-containing fractions were pooled and concentrated by ultrafiltration (Amicon Ultra-15; Millipore).

2.4. Immunoblot analysis

Proteins were separated either by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% gels or by native PAGE using 4–15% gradient gels and subsequently transferred to Protran BA85 nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany). The RH subunits were identified using anti-HoxC serum, diluted 1:1000, and anti-HoxB serum, diluted 1:10,000. Polyclonal antibodies raised against the Hyp proteins from *R. eutropha* were used in the following dilutions: HypB 1:500, HypC 1:1000, HypD 1:500, HypE 1:500, HypF 1:5000, HypX 1:500. The second antibody was alkaline-phophatase labeled goat anti-rabbit IgG (Dianova, Hamburg, Germany).

2.5. Analytical techniques

Metal analysis by atomic absorption spectroscopy (AAS) and total reflection X-ray fluourescence (TRXF) analysis was performed as in [8], mass determination by analytical size exclusion chromatography as in [15]. H_2-oxidizing activity was determined by an amperometric H_2-uptake assay [16]. The in-gel activity staining assay was described in [17]. Protein concentration was determined according to [18].

2.6. MALDI-TOF

Coomassie-stained protein bands were cut from an SDS–PAGE and washed four times with 40% acetonitrile to remove the Coomassie reagent. The gel slices were dehydrated by treatment with 100% acetonitrile and subsequent vacuum drying. Proteins were digested overnight at 37 °C in a solution of 5 mM ammonium bicarbonate containing 10 μg/ml trypsin (sequencing grade modified trypsin, Promega). 1.5 μl of the digest was mixed with 1 μl of the matrix dihydroxybenzoate (5 mg/ml in 0.1% trifluoracetic acid) and applied to the sample target (AnchorChip™). Mass spectra were recorded with a Reflex IV MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). External calibration with peptide standards was used. For protein identification, a database search was performed with the NCBI protein database by using the Mascot Peptide Mass Fingerprint program (http://www.matrixscience.com).

3. Results

Expression of the RH large subunit, HoxC, is based on the P_{SH}-*hoxB-hoxC* unit in which the RH structural genes are fused to the strong inducible SH promoter. This unit has already been employed in the homologous overexpression of RH in *R. eutropha* [12]. In this study, a *Strep*-tag II coding sequence was fused to the start codon of *hoxC* to yield an N-terminal *Strep*-tag on the RH large subunit ($HoxC_{ST}$). Second, an in-frame deletion of 45% of the *hoxB* gene was generated. The resulting construct was transferred to *R. eutropha* HF574, a strain devoid of hydrogenases.

To examine whether $HoxC_{ST}$ was stably expressed in the absence of HoxB, soluble protein extracts were analyzed for the presence of the RH by immunoblot analysis. HoxC was detected in extracts derived from the wild-type as well as in extracts from the $HoxC_{ST}$ strain (Fig. 1A, lanes 1 and 2). The migration of HoxC was consistent with the predicted molecular masses of the wild-type HoxC (52.4 kDa; lane 1) and the *Strep*-tag-containing derivative (53.4 kDa; lane 2). Since both HoxC-bands were of similar intensity, we conclude that $HoxC_{ST}$ was stably expressed in *R. eutropha*. The absence of the HoxB subunit in extracts of $HoxC_{ST}$ was corroborated by HoxB-specific immunoblot analysis (Fig. 1B, lane 2).

$HoxC_{ST}$ was purified from extracts of HF574(pGE537) by *Strep*-tag affinity chromatography and gel filtration (see Section 2). Notably, two elution peaks obtained from size exclusion chromatography contained predominantly $HoxC_{ST}$ as verified by SDS–PAGE analysis (data not shown). Thus, we concluded that two different forms of $HoxC_{ST}$ were purified. By comparing the retention times of the two $HoxC_{ST}$-containing pools with those of standard proteins (Fig. 2), the molecular mass of the heavier form of $HoxC_{ST}$ (form 1) was estimated to be 152 kDa, whereas the mass of the lighter form (form 2) was determined to be 96 kDa. The amino acid sequence of $HoxC_{ST}$ predicts a mass of 53.4 kDa. Thus, the lighter form may be a dimer of $HoxC_{ST}$. The heavier form may be either a trimer of $HoxC_{ST}$ or a complex of $HoxC_{ST}$ dimer and additional proteins (see below). All complexes were stable in the presence of dithiol-reducing reagents such as tris(2-carboxyethyl)-phosphine [TCEP (data not shown)], indicating that disulfide bonds are not involved in complex formation.

Metal analysis by AAS and TRXF revealed that both forms of $HoxC_{ST}$ contained approximately one Fe per $HoxC_{ST}$ monomer, as expected. However, the amount of Ni was substoichiometric; the Ni/Fe ratio was 0.44 for form 1 and 0.63

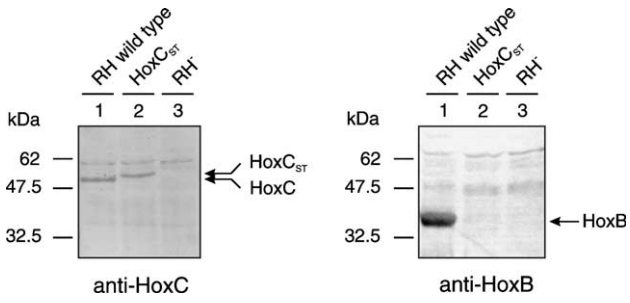

Fig. 1. Immunological detection of the RH subunits in *R. eutropha* soluble extracts. Proteins were separated by SDS–PAGE (12% gels; 30 μg of protein per lane). Lane 1, HF574 (pGE377) [12,13]; lane 2, HF574 (pGE537); lane 3, HF574 (pEDY309).

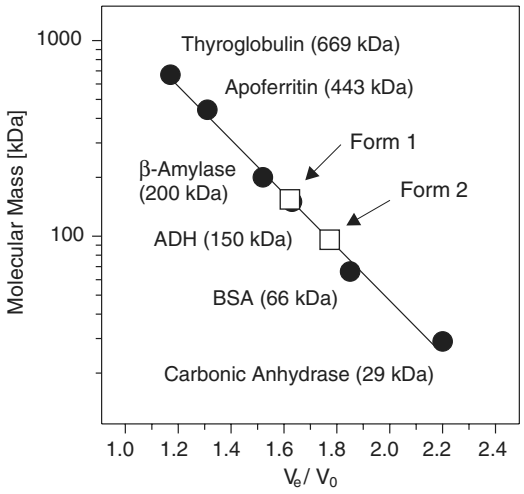

Fig. 2. Mass determination by size exclusion chromatography. The molecular mass of the two forms of $HoxC_{ST}$ (open squares) obtained after gel filtration was calculated from their retention times (elution volume/void volume (V_e/V_0)) in comparison to those of standard proteins (filled circles; molecular weight marker kit MW-GF-1000, Sigma) as indicated in the figure. The data shown are the average of two independent experiments.

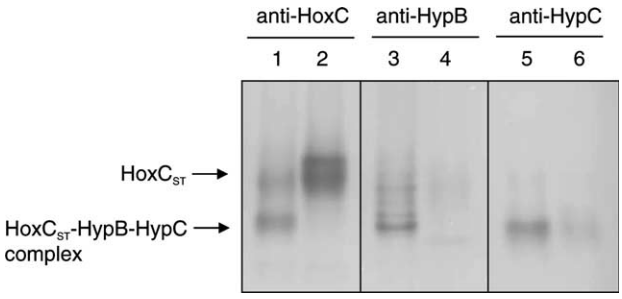

Fig. 3. Immunological analysis of the two $HoxC_{ST}$ forms separated on non-denaturing 4–15% gradient gels (15 μg of protein per lane). Form 1 was applied to lanes 1, 3 and 5; form 2 was applied to lanes 2, 4 and 6. The gel was subsequently blotted and the membrane was cut and developed using different antisera as indicated above the figure.

for form 2. These data indicate that the Fe-site was fully occupied; however, Ni was incompletely incorporated into both forms.

To determine whether the $HoxC_{ST}$ preparations could activate hydrogen, both forms were analyzed by a number of biochemical assays commonly used to measure the H_2- oxidizing activity of wild-type RH. $HoxC_{ST}$, however, was inactive in the amperometric H_2 uptake assay, using either methylene blue or ferricyanide as the artificial electron acceptor, as well as in an in-gel activity staining using phenazine methosulfate as artificial electron acceptor. This assay is highly sensitive to low level hydrogenase activity [17]. Finally, $HoxC_{ST}$ was also inactive in H^+/D_2 exchange [6] (data not shown). In summary, under all conditions tested, $HoxC_{ST}$ did not oxidatively cleave dihydrogen.

The low amount of Ni in both forms of $HoxC_{ST}$ indicated incomplete metal center assembly into the protein. Both in general and in the specific case of RH, the assembly of the Ni–Fe active site is a complex process involving at least six accessory proteins: HypA-HypF [19]. Thus, the high molecular weight complex from form 1 may represent a maturation intermediate of $HoxC_{ST}$ involving one or more Hyp proteins. To establish whether any of the Hyp proteins could be detected in complexes with the purified $HoxC_{ST}$, the proteins of both pools were separated by native PAGE. Immunoblot analysis revealed that both pools contained $HoxC_{ST}$ (Fig. 3, lanes 1 and 2). In case of pool 1, but not pool 2, the HoxC-specific band also cross-reacted with HypC and HypB antisera (Fig. 3, lanes 3–6). We conclude that these three proteins form a complex. Both samples were also examined for the presence of HypD, HypE and HypF as well as HypX, an additional Hyp protein present in aerobic organisms such as *R. eutropha* [20]. However, neither pool contained detectable amounts of any of these proteins (data not shown). Due to the lack of a HypA-specific antibody, MALDI-TOF was employed to examine whether HypA participates in the protein complex of form 1. Proteins of form 1 were separated by SDS–PAGE and several gel slices were cut from the region of 6 kDa to about 25 kDa and analysed by MALDI-TOF. HypC (9.8 kDa) was clearly identified using this technique; however, no evidence was obtained for the presence of HypA (12.8 kDa) in the isolated protein complex of form 1 (data not shown).

4. Discussion

The large and small subunits of [NiFe] hydrogenases are tightly bound via a large, shared, hydrophobic surface. Therefore, the heterodimer is generally regarded as an inseparable, functional unit. In this study, we demonstrate that the large subunit HoxC of the H_2-sensing [NiFe] hydrogenase of *R. eutropha* can stably exist in the absence of its associated small subunit. HoxC was purified as a *Strep*-tag fusion protein from a background lacking the corresponding small subunit HoxB. Two different forms of $HoxC_{ST}$ were identified, one of which might be a $(HoxC_{ST})_2$ dimer. Although about half of the $HoxC_{ST}$ units carry the bimetallic active site, the second half of the protein sample is lacking nickel. This indicates that either the assembly of the Ni–Fe site is inefficient in $HoxC_{ST}$ or that nickel is not stably inserted into $HoxC_{ST}$ maybe due to some general stability problem. Since dimeric RH has been shown to contain one nickel per protein, the lack of the small subunit HoxB seems to have an effect on proper nickel incorporation into or retention in $HoxC_{ST}$ [8]. We further note that even the portion of $HoxC_{ST}$ that contained a complete active site was nonetheless incapable of activating H_2, indicating that HoxB is essential for catalytic activity. It is commonly believed that at least one Fe–S cluster is needed for catalytic activity since it receives one electron that is released by the Ni–Fe site upon its reaction with H_2 [21]. Thus, our findings corroborate the hypothesis that the mechanism of hydrogen oxidation by the active site actually requires at least one Fe–S cluster of the small subunit.

The second form of $HoxC_{ST}$ is a larger complex that includes the accessory proteins HypB and HypC. The experimentally determined mass of 152 kDa agrees with the calculated mass of 155 kDa for a $(HoxC_{ST})_2$–HypB–HypC complex. The low Ni/Fe ratio suggests that the $HoxC_{ST}$ subunits which have not yet undergone nickel incorporation are still coordinated by the maturation proteins HypB and HypC. HypB homologs from *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*

have been shown to bind nickel ions in vitro [22,23]. Thus, it has been suggested that HypB has a function in nickel storage and nickel delivery to the hydrogenase apoprotein. In contrast, the HypB homolog from *Helicobacter pylori* does not bind nickel ions [24]. It was shown that HypA in concert with HypB mediates nickel incorporation in *H. pylori* as well as in *E. coli* [24,25]. In case of *R. eutropha*, the precise roles of HypA and HypB have not been studied in detail; however, the data presented here suggest that only HypB forms a stable complex with $HoxC_{ST}$, whereas HypA was not identified within this complex. Moreover, the low Ni/Fe ratio of the complex indicates that HypB, as present in the isolated form 1, does not obviously carry excess nickel ions. Our data can not differentiate whether the nickel is bound to $HoxC_{ST}$ or HypB in form 1. The spectroscopic data of the accompanying manuscript, however, show that the Ni coordination environments of both forms of $HoxC_{ST}$ are identical [9], suggesting that nickel is located in $HoxC_{ST}$ in both forms 1 and 2.

Complex formation between a [NiFe] hydrogenase large subunit and HypC has been demonstrated for *E. coli* hydrogenase 3 as well as for the SH from *R. eutropha* and for one of the two membrane-bound hydrogenases from *Thiocapsa roseopersicina* [26–28]. Within these complexes, HypC is believed to function as a chaperone, keeping the hydrogenase large subunit in a conformation competent to receive the active site components incorporated by other Hyp proteins. Recently, a HoxC-HypC complex has also been identified [29]. The mechanism of Hyp-assisted metal center assembly has predominantly been studied in *E. coli*. According to these data, the $Fe(CO)(CN)_2$ moiety is first pre-synthesized on a HypCD complex by HypE and HypF [19,30,31]. Based on immunochemical data, a similar model for the interactions between HypC, HypD, HypE and HypF has recently been established for *R. eutropha* [32]. Thus, HypC has a dual function within these two systems, whereas in *T. roseopersicina* the two functions seem to be separated over two different HypC proteins, $HypC_1$ and $HypC_2$ [33]. One of them ($HypC_1$) is putatively involved in the synthesis of the Fe fragment in a complex with HypD, whereas the second one ($HypC_2$) functions as a chaperone for the immature hydrogenase large subunit [28]. In *R. eutropha* it is not yet clear whether two distinct copies of HypC participate in the maturation process or whether one copy shuttles between different complexes. Although the lack of detection of a $HoxC_{ST}$-HypC-HypD supercomplex might tend to suggest that two copies of HypC are necessary, one for interaction with HypD and another for interaction with the immature large subunit, it could be that this complex is simply too unstable to be isolated using the current techniques.

Nickel is believed to be incorporated after Fe-fragment addition by HypA and HypB [19,34]. In this study, we show for the first time that HypB forms a complex with a hydrogenase large subunit. Interestingly, HypC but not HypA is also present in this complex. These new results perhaps remind us that the question of the mechanism of Ni incorporation into [NiFe] hydrogenases is still open. Detection of a HoxC form containing Fe but not Ni also provides direct evidence for the consecutive incorporation of the $Fe(CO)(CN)_2$ unit and then the Ni atom.

In conclusion, isolation of $HoxC_{ST}$ in the absence of HoxB has proven a useful tool for a detailed spectroscopic analysis of the [NiFe] active site in the absence of the iron sulfur clusters normally present in the small subunit [9]. Moreover, our findings of the present study conclusively demonstrate that the Fe fragment of the active site is present in the large subunit before Ni incorporation, making $HoxC_{ST}$ also an ideal model system for further studies of hydrogenase maturation.

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