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Carbamoylphosphate serves as the source of CN^-, but not of the intrinsic CO in the active site of the regulatory [NiFe]-hydrogenase from *Ralstonia eutropha*

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Abstract Within the catalytic centre of [NiFe]-hydrogenases one carbonyl and two cyanide ligands are covalently attached to the iron. To identify the metabolic origins of these ligands, the regulatory [NiFe] hydrogenase in conjunction with the indigenous Hyp maturation proteins of *Ralstonia eutropha* H16 were heterologously overproduced in *E. coli* grown in the presence of L-[ureido-^{13}C] citrulline and $NaH^{13}CO_3$. Infrared spectroscopy of purified hydrogenase provided direct evidence that only the cyanide ligands, but not the CO ligand, originate from CO_2 and carbamoylphosphate. Incorporation of label from ^{13}CO exclusively into the carbonyl ligand indicates that free CO is a possible precursor in carbonyl ligand biosynthesis.

Keywords: Regulatory hydrogenase; Fourier-transform infrared spectroscopy; Metallocentre assembly; Nickel; Iron; Metal carbonyl; Cyanide

1. Introduction

H_2 oxidation and proton reduction catalysed by [NiFe] hydrogenases take place at the active site deeply buried within the large subunit of these enzymes. The catalytic centre displays a unique architecture that involves the two metal ions, nickel and iron, which are coordinated by the thiolates of four highly conserved cysteine residues. The iron carries three diatomic ligands, two cyanides and one carbon monoxide, which maintain the metal in the low-spin state [1–3]. The complexity of this architecture is reflected in an elaborate maturation machinery involving at least six specific proteins that are required for the proper assembly of the Ni–Fe active site (recently reviewed in [4–6]).

Genetic and physiological studies using *Escherichia coli* mutants with lesions in the *carAB* genes encoding carbamoylphosphate synthetase, revealed that carbamoylphosphate is essential for the synthesis of functional hydrogenases in this organism [7]. Results of protein chemistry and mass spectrometry confirmed that carbamoylphosphate is activated in an ATP-dependent manner by the HypF protein. During the reaction the carbamoyl group is transferred to HypE giving rise to HypE-thiocarboxamide that is further dehydrated to HypE-thiocyanate by means of ATP hydrolysis [8]. Only HypE carrying the cyanate moiety was able to transfer the CN^- to the HypCD complex [9,10]. There is only indirect evidence that cyanide is further transferred from HypCD to the large subunit of hydrogenase 3 from *E. coli* [9,11]. It has been noted that the iron-bound CO could, in principle, also stem from carbamoylphosphate [7]. However, more than 7 years ago, labelling experiments with $NaH^{13}CO_3$ in *Allochromatium vinosum* revealed an inhomogeneous incorporation of the label into the CN^- and CO ligands of the indigenous membrane-bound hydrogenase, as determined by Fourier-transform infrared (FTIR) spectroscopy [12]. This was surprising in view of the fact that *A. vinosum* is able to replenish its organic carbon pool by assimilation of CO_2 via the Calvin–Benson–Bassham cycle. From these observations it was assumed that CN^- and CO may have different metabolic origins. This assumption was supported by recent labelling experiments in *A. vinosum* with ^{13}C-labelled acetate as an additional carbon source from which the carboxy-carbon was partially incorporated into the CO ligand, but not into the CN^- ligands [10].

To provide direct evidence that the diatomic ligands of hydrogenases derive from different sources, we took advantage of the observation that lack of carbamoylphosphate for hydrogenase synthesis in a *carAB* mutant of *E. coli* can be rescued by the addition of citrulline to the medium. Although thermodynamically unfavourable, citrulline is converted to carbamoylphosphate and ornithine by the ornithine transcarbamylase ArgI, especially if the ArgI protein is overproduced [11]. This made it possible to use L-[ureido-^{13}C] citrulline for labelling studies.

In this communication, the cytoplasmic regulatory hydrogenase (RH) of *Ralstonia eutropha* H16 was chosen as a model for labelling experiments for the following reasons: (i) The *E. coli* hydrogenases, particularly hydrogenase 3, are extremely difficult to purify in sufficient quantities for infrared spectroscopy; (ii) the RH contains a catalytic centre with a diatomic ligand pattern similar to that of standard [NiFe]-hydrogenases [13,14]; (iii) the RH shows clear infrared signals for the CN^- and CO ligands, even at low protein concentrations; and (iv),

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the RH displays only two distinct states in infrared spectroscopy, an as-isolated, oxidised and a reduced state, which significantly facilitates the interpretation of FTIR spectra [13–15].

The RH was heterologously overproduced in the *E. coli* wild-type and a Δ*carAB* derivative. Catalytically active RH was purified from the *E. coli* cells cultivated with either L-[ureido-^{13}C] citrulline, $NaH^{13}CO_3$ or ^{13}CO. FTIR spectroscopy revealed that the CO ligand originated from a different source than the CN^- ligands, which are derived from CO_2-borne carbamoylphosphate. The data are in agreement with the results presented in the accompanying paper by Forzi and co-workers which deals with the selective labelling of the CN^- ligands of *E. coli* hydrogenase 2 by L-[ureido-^{13}C] citrulline on the one hand and the carbonyl ligand by ^{13}CO on the other hand.

2. Materials and methods

2.1. Strain and plasmids

E. coli JM109 [16] served as host for plasmid constructions. For labelling studies strain *E. coli* MC4100 (wild-type) and its derivatives DcarAG (Δ*carAB*, Δ*argG*), BEF314 (Δ*hyp[BCDE]*) and DHPF2 (Δ*hypF*), were used [11,17,18]. The RH-encoding genes *hoxB*$_{stop}$-*Strep*-tagII and *hoxC* were amplified by PCR using primers 5′-GGAGCCATGGGCGCGCCTGTATGTACCGGTC-3′ and 5′-GTGAAGCTTGAGGTGCATGCTCAATGCACG-3′ and pGE567 [15] as template. A 2.52 kbp NcoI–HindIII DNA fragment was cloned into pQE-60 [Quiagen, Hilden] resulting in pRH which expresses the RH genes under control of the T5 promoter. The *R. eutropha hypA1B1F1CDE* genes controlled by the *lac* promoter were inserted as a 7.82-kbp SspI fragment into the single MscI site of pRH. From the resulting plasmid a 12.12-kbp AseI–XbaI fragment carrying the RH structural and *hyp* genes was transferred to the vector pCM62 [19], giving rise to pRH-Hyp. pRH-Hyp(ΔF) is a derivative of pRH-Hyp with a 477-bp NarI inframe deletion in *hypF1*. pBargI which overexpresses *argI* encoding ornithine carbamoyltransferase [11] was co-transformed together with pRH-Hyp(ΔF) when indicated.

2.2. Media and growth conditions

E. coli cells were grown semi-anaerobically at 30 °C in 5-L Erlenmeyer flasks sealed with parafilm for 24 h under continuous rotation at 40 rpm. The basic glucose–ammonium (GN) minimal medium contained 25 mM Na_2HPO_4 and 11 mM KH_2PO_4 (pH 7.0), 37 mM NH_4Cl, 0.8 mM $MgSO_4$, 68 μM Ca_2Cl, 18 μM $FeCl_3$, 1 μM $NiCl_2$ and 20 mM glucose. Medium for *E. coli* DcarAG (pRH-Hyp(ΔF), pBargI), additionally contained 10 μg/mL tetracyclin, 50 μg/mL ampicillin and was supplemented with 50 μg/mL of arginine and 50 μg/mL of uracil. For labelling experiments cells were grown in the presence of either 1 μg/mL L-[ureido-^{13}C] citrulline (99% ^{13}C, Cambridge Isotope Laboratories), 5 mM $NaH^{13}CO_3$ (99% ^{13}C, from Cambridge Isotope Laboratories). For labelling with ^{13}CO (99% ^{13}C, <5% ^{18}O, Sigma–Aldrich) in the gas phase, cells were grown in rubber-stoppered serum bottles containing 1 L medium and 3.1% (vol/vol) carbon monoxide in the 160 mL gas phase.

2.3. Purification of Strep-tagged RH

Strep-tagged RH was purified from 3 to 4 g cells (wet weight). The cell pellet was resuspended in buffer A (100 mM Tris–HCl, pH 8.0, 150 mM NaCl; 1 mL buffer A per 1 g of cells), and passed twice through a French pressure cell at 900 psi. Cell debris was removed by centrifugation for 45 min at 90000 × g at 4 °C. The soluble extract was applied to a *Strep*-Tactin Superflow column (1 mL bed volume, IBA, Göttingen, Germany) which was run by gravity flow. The column was washed with 10 column volumes (CV) of buffer A. Proteins were eluted with 5 CV of buffer containing 5 mM desthiobiotin. Protein-containing fractions were pooled and concentrated by ultrafiltration using Amicon Ultra-15 and Microcon devices (Millipore) to obtain volumes less than 50 μL.

2.4. Fourier transform infrared spectroscopy

Infrared spectra were recorded on a Bruker IFS66V/S spectrometer equipped with a liquid nitrogen-cooled MCT detector at a spectral resolution of 2 cm^{-1}. The sample compartment was purged with nitrogen, and the sample was held in a temperature-controlled (23 °C) gas-tight liquid cell (volume 7 μL, path length = 50 μm) with CaF_2 windows. Spectra were baseline-corrected by using a spline function implemented within OPUS 4.2 software supplied by Bruker. Reduced protein samples were prepared through incubation under 100% H_2 for 30 min at room temperature.

3. Results

3.1. Construction of a plasmid for heterologous production of the regulatory hydrogenase

Attempts to perform labelling experiments with L-[ureido-^{13}C] citrulline in *R. eutropha* failed so far, because it turned out not to be possible to inactivate genetically the carbamoylphosphate synthetase readily, most probably due to the fact that, besides the requirement for arginine and pyrimidine biosynthesis, other key pathways in *R. eutropha* require carbamoylphosphate. Consequently, the RH from *R. eutropha* was heterologously overproduced in *E. coli* for which *carAB* mutants were already available [7,11].

In order to obtain an efficient RH production plasmid, the genes encoding the so-called RH$_{stop}$ protein [15], consisting of the large subunit HoxC and the small, C-terminally truncated HoxB subunit, equipped with a C-terminal *Strep*-tag, were subcloned into plasmid pQE-60 under the control of the T5 promoter. The plasmid was transformed into *E. coli* MC4100 and the resulting recombinant was grown in GN minimal medium under either aerobic or anaerobic conditions. HoxB and HoxC were immunologically detected in relatively high amounts using specific antibodies raised against the native RH$_{stop}$ protein. However, H_2-dependent reduction of PMS in native PAGE gels revealed absolutely no RH activity, even under anaerobic conditions (data not shown), indicating that the Hyp proteins of *E. coli* are not capable of restoring the function of their *R. eutropha* counterparts. Thus, we established the *lac* promoter-driven *hypA,B,F,C,D,E* genes of *R. eutropha* on the pRH plasmid giving rise to pRH-Hyp. *E. coli* MC4100 cells harboring pRH-Hyp produced catalytically active RH as determined by in-gel activity staining (Fig. 1). Thus, for the first time a catalytically active [NiFe]-hydrogenase was heterologously produced in *E. coli*. Surprisingly, RH activity was observed exclusively in anaerobically cultivated cells in rather low amounts. This result was unexpected since the RH should be

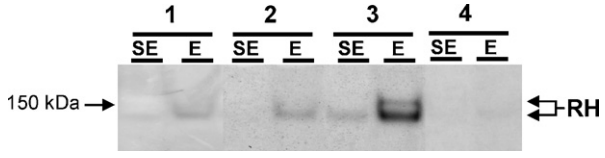

Fig. 1. Activity of *R. eutropha* regulatory [NiFe]-hydrogenase heterologously produced in *E. coli*. Plasmid-harbouring *E. coli* strains were grown in GN medium. The cells were collected, disrupted and the resulting soluble extracts subjected the *Strep*-Tactin affinity chromatography. Twenty micrograms of soluble extract (SE) and 2 μL of the pooled eluate (E) were applied to a 3–30% native PAGE gel. In-gel hydrogenase activity was determined as described [14]. 1, *E. coli* MC4100 (pRH-Hyp); 2, *E. coli* BEF314 (pRH-Hyp); 3, *E. coli* MC4100 (pRH-Hyp[ΔF]). 4, *E. coli* DHPF2 (pRH-Hyp).