Functional roles of HypC and HupK in the biosynthesis of [NiFe] hydrogenase in *Rhizobium leguminosarum.*

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ABSTRACT

Some rhizobia induce a hydrogen (H_2)-uptake system with a [NiFe] hydrogenase along with nitrogenase to recover part of the energy lost as H_2. Biosynthesis of NiFe hydrogenases is a process that ocurrs in the cytoplasm, where a number of auxiliary proteins (products of *hup* and *hyp* genes) are required to synthesize and insert the metal cofactors into the enzyme structural units. Although HypC is expressed in all hydrogenase systems, HupF and HupK are found only in bacteria that express the hydrogenase in the presence of oxygen (O_2). Co-purification experiments have demonstrated HypC-HupK and HypC-HupL interactions. Results have shown that some conserved residues from HypC and HupK play a protective role of hydrogenase against the presence of O_2.

INTRODUCTION

Rhizobial hydrogenase is a membrane-bound enzyme that contains two subunits, a large subunit containing the active center of the enzyme $NiFe(CN^-)_2CO$ and a small subunit that accomodates three Fe-S clusters. In *R. leguminosarum* bv. viciae 18 genes (*hupSLCDEFGHIJKhypABFCDEX*) clustered in the symbiotic plasmid are required for hydrogenase biosynthesis (Ruiz-Argüeso *et al*., 2001). Analysis of *Escherichia coli* hydrogenase-3 has demonstrated that $Fe(CN^-)_2CO$ cofactor intermediate is synthesized through the concerted action of HypC-HypD complex with HypF and HypE. When the iron is fully coordinated, HypC transfers the precursor cofactor to pre-HupL (Böck *et al.*, 2006). The *R. leguminosarum* cluster encodes two proteins (HupF and HupK) not present in those systems in which hydrogenase is synthesized under anaerobic conditions, such as *E.coli*. HupF acts as a chaperone to stabilize HupL when hydrogenase is synthesized in the presence of O_2 (Albareda *et al*., 2012). HupK is a scaffolding protein for the transferring of precursor cofactor to HupL (Ludwig *et al*., 2009). In this work we have studied the functional role of HypC and HupK in the biosynthesis of hydrogenase in *R. leguminosarum.*

MATERIAL AND METHODS

In frame deletions of *hypC* and *hupK* genes were generated in plasmid pALPF1 (Brito *et al*., 2002). This plasmid drives the expression of hydrogenase cluster under the FnrN-dependent *fixN* promoter (P*fixN*) allowing expression of hydrogenase in microaerobic vegetative cells. The resulting plasmids were transferred by conjugation into Hup^- *R. leguminosarum* UPM1155 strain. HypC and HupK protein variants incorporating affinity tags, HypC-StrepTag ($HypC_{ST}$) and HupK-StrepTag ($HupK_{ST}$), expressed from a pBBR1MCS-based plasmid, were constructed as described in Albareda *et al*. (2012). $HypC_{ST}$ and $HupK_{ST}$ altered in different residues were generated by site-directed mutagenesis using a PCR-assisted codon replacement strategy. Hydrogenase activity was induced in vegetative cells grown under continuous bubbling with a gas containing 1% O_2 in N_2 and was measured by an amperometric method with O_2 as final acceptor.

RESULTS AND DISCUSSION

Hydrogenase activity of *hypC*- and *hupK*-deleted mutant strains was drastically reduced. These data indicate that both proteins are essential for hydrogenase biosynthesis. Co-purification experiments using $HypC_{ST}$ have demonstrated a direct interaction between HypC and HupK. Furthermore, these experiments have revealed the existence of a complex involving HypC and HupL in a HupK-dependent manner.

The functional role of some conserved residues of $HypC_{ST}$ (C2S, L33A, H46A and E58L) was studied. Hydrogenase activity of microaerobic (1% O_2) cultures expressing $HypC_{ST}$ variants C2S, L33A and H46A was similar to that exhibit by the negative control (Δ*hypC*). In contrast, $HypC_{ST}$ variant E58L complemented the Δ*hypC* mutant strain to wild type levels. Immunoblot analysis showed that crude extracts of C2S, L33A and H46 mutants accumulated the unprocessed form of HupL. Under symbiotic conditons, $HypC_{ST}$ L33A strain complemented the Δ*hypC* mutation, and consistently, HupL was processed. This result indicates that L33 might be necessary for the adaptation of hydrogenase to the presence of O_2. Co-purification experiments have shown that these residues are involved in the HypC-HupK interaction.

In free-living microaerobic conditions, HupK variants affected in residues participating in the precursor cofactor binding site (C22, C57, F54, C357 and F360) showed hydrogenase-deficient phenotype, whereas the alteration of other residues potentially involved in HypC-HupK interaction had no significant effect on hydrogenase maturation. In contrast, hydrogenase levels detected in symbiotic cells were similar to those in the wild type strain in all the cases. These results might indicate that the HypC->HupK->HupF->HupL pathway for cofactor transfer might coexist in bacteroids with a HupK-independent pathway similar to that described in *E. coli*.

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