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Dissection of the maturation reactions of the [NiFe] hydrogenase 3 from *Escherichia coli* taking place after nickel incorporation

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Abstract The steps in the maturation of the precursor of the large subunit (pre-HycE) of hydrogenase 3 from *Escherichia coli* taking place after incorporation of both iron and nickel were investigated. Pre-HycE could be matured and processed in the absence of the small subunit but association with the cytoplasmic membrane required heterodimer formation between the two subunits. Pre-HycE formed a complex with the chaperone-like protein HypC in the absence of the small subunit and, in this complex, also incorporated nickel. For the C-terminal processing, HypC had to leave the complex since only a HypC-free, nickel-containing form of pre-HycE was a substrate for the maturation endopeptidase.

Key words: [NiFe] hydrogenase; Maturation; Cellular location; *Escherichia coli*

1. Introduction

A detailed analysis of genes controlling the synthesis of catalytically active [NiFe] hydrogenases has discriminated four functional categories, namely (i) the structural genes [1], (ii) genes encoding metal transport components [2–4], (iii) genes whose products are involved in the biosynthesis and insertion of the complete metal cluster [5] and (iv) genes whose products translocated the fully maturated enzyme through the cytoplasmic membrane in the case of those hydrogenases located at the periplasmic side [6–10]. The analysis of those gene products with a function in enzyme maturation has revealed a surprisingly complex assembly of proteins with different roles, amongst others a chaperone-type protein [11], a GTPase [12] and a maturation endopeptidase [13]. A model has been presented which implies that (i) iron and nickel are inserted separately into the apoprotein of the large subunit of the hydrogenase and (ii) the precursor containing both metals is C-terminally cleaved which triggers a conformational switch in which the free thiol of the most C-terminally located cysteine residue closes the bridge between the two metals resulting in the formation of the complete heterobinuclear center [14–16].

The fully maturated and active hydrogenase is a heterodimer made up of the large subunit which carries the [NiFe] cluster and the electron transferring small subunit which, depending on the organism, contains one or more iron–sulfur clusters [17–20]. As disclosed by the crystal structure of the *Desulfovibrio gigas* [NiFe] hydrogenase, the two subunits are intimately connected over a common surface of 3500 Å^2 [17]. One of the open issues in our knowledge on the maturation process is whether the insertion of the metal and the proteolytic maturation occur at the free large subunit precursor polypeptide or whether it requires the prior association with the small subunit. A second open issue concerns the sequence of the individual maturation steps. It was shown that a key intermediate during maturation is a complex between the precursor of the large subunit and a chaperone-type protein, HypC in the *Escherichia coli* nomenclature. It was unknown until now whether the maturation endopeptidase acts on this complex, possibly leading to release of HypC, or whether this complex has to be resolved before proteolysis takes place. In this communication, we show for the *E. coli* hydrogenase 3 that (i) maturation can take place at the free large subunit and (ii) the chaperone-specific protein has to leave the complex with the precursor of the large subunit before the maturation endopeptidase can act.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are derivatives of *E. coli* MC4100 [21], namely HD705 (MC4100, Δ*hycE*) [22], HD707 (MC4100, Δ*hycG*) [22], HD709 (MC4100, Δ*hycI*) [23], DHP-C (MC4100, Δ*hypC*) [24], DHP-D (Δ*hypD*) [24] and HYD723 (MC4100, *nikA*::MudI) [2]. The *E. coli* strains were cultivated under anaerobic conditions at 37°C in TGYEP as previously described [11]. Growth of strain HYD723 occurred either in the presence of 50 μM EDTA to chelate the nickel in the medium or in the presence of 400 μM of nickel chloride to restore the wild-type phenotype.

2.2. Cell fractionation and in vitro processing assays

Following addition of a 1% inoculum, cultures were grown at 37°C until they reached an optical density of 1.0 at 600 nm. Cells were harvested by centrifugation and washed in 50 mM Tris–HCl pH 7.4, 1 mM dithiothreitol. Soluble and membrane fractions obtained by ultracentrifugation at 100 000×*g* (named S100 and P100 extracts, respectively) were prepared as previously described [11]. Triton X-100-dispersed membranes were applied onto the gels. The processing assays were conducted with purified HycI endopeptidase under anaerobic conditions at 37°C as reported previously [25,26]. Electrophoresis on non-denaturing gels was conducted as previously described [11].

2.3. Nickel incorporation experiments

Analysis of in vivo nickel incorporation into the large subunit of hydrogenase 3 was performed as described [14,27] using 150 nM of ^{63}Ni (specific radioactivity 190 μCi/μmol) in the growth medium.

3. Results and discussion

3.1. Cellular localization of the large subunit during processing

Hydrogenase 3 from *E. coli* is part of the formate–hydro-

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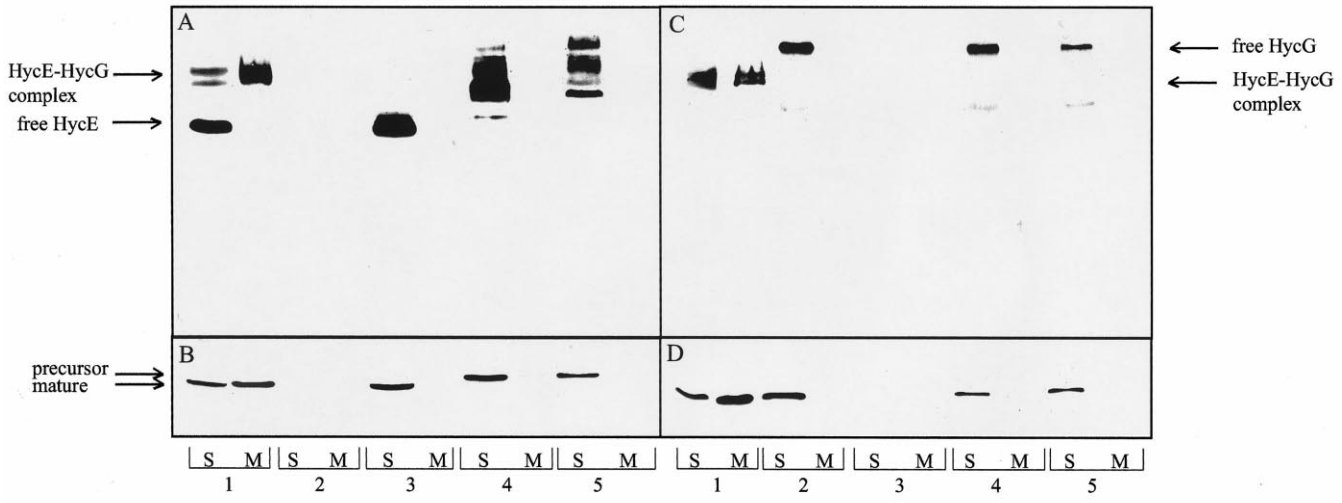

Fig. 1. Cellular localization of HycE and HycG. Soluble (S) and membrane (M) fractions (30 μg of total protein) were subjected to SDS–PAGE (B and D) or to non-denaturing PAGE (A and C) and reacted with antibodies raised against HycE (A and B) and HycG (C and D). Extracts from the following strains were applied on the gel: MC4100 (lanes 1), HD705 (Δ*hycE*) (lanes 2), HD707 (Δ*hycG*) (lanes 3), HD709 (Δ*hycI*) (lanes 4) and DHP-C (Δ*hypC*) (lanes 5). Characteristic bands are denoted.

genlyase complex which consists of the large and small hydrogenase subunits HycE and HycG, respectively, several electron carriers (HycB and HycF) and two membrane integral proteins (HycC and HycD) [22]. It has been demonstrated previously that, at least, the mature large subunit is membrane-associated and can be detached upon breakage of the cells with the French press under high pressure conditions [22].

To investigate the cellular localization of the large and small subunit during maturation, we have followed their distribution between the $100\,000 \times g$ supernatant (S100) and the membrane fraction (P100) from the wild-type and from mutants with in-frame deletions either in the *hycE* (HD705) or *hycG* (HD707) gene [22]. The proteins were separated by SDS–PAGE and non-denaturing PAGE and the migration of HycE and HycG was analyzed by immunoblotting (Fig. 1). HycE and HycG from wild-type cells show the approximate 1:1 distribution already described [22] (Fig. 1B,D). Upon non-denaturing PAGE, HycE from the S100 migrates in two bands (Fig. 1A, lane 1), one representing free HycE because no antiserum directed against other formate–hydrogenlyase complex components showed a reaction with it (data not shown). The other, slower migrating band reacts with anti-HycG serum, indicating that it represents a HycE–HycG complex. Both free and complexed HycE were processed (Fig. 1B). When extracts from the Δ*hycE* strain HD705 were analyzed (Fig. 1, lane 2), all HycG cross-reacting material was in the soluble fraction and its migration was retarded in the non-denaturing PAGE (Fig. 1C). It is assumed that this band represents free HycG since no other antiserum directed

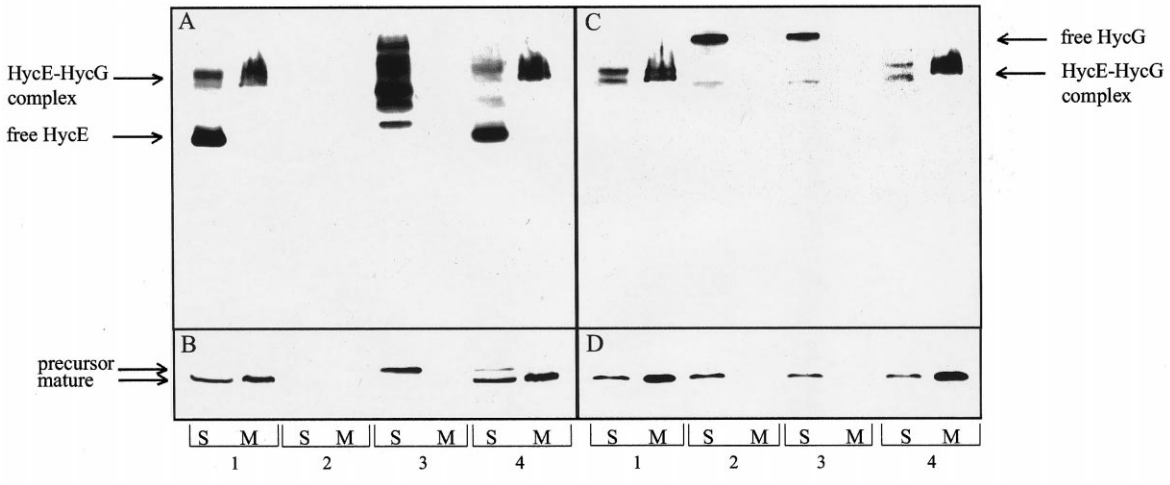

Fig. 2. Effect of nickel on the cellular localization of HycE and HycG. Soluble (S) and membrane (M) fractions (30 μg of total protein) were analyzed by Western blotting after SDS–PAGE (B and D) or non-denaturing PAGE (A and C) with antisera directed against HycE (A and B) and HycG (C and D). Extracts from the following strains were applied on the gel: MC4100 (lanes 1), HD705 (Δ*hycE*) (lanes 2), HYD723 (*nik*$^-$) grown in the absence (lanes 3) or presence (lanes 4) of 400 μM nickel. Characteristic bands are marked.

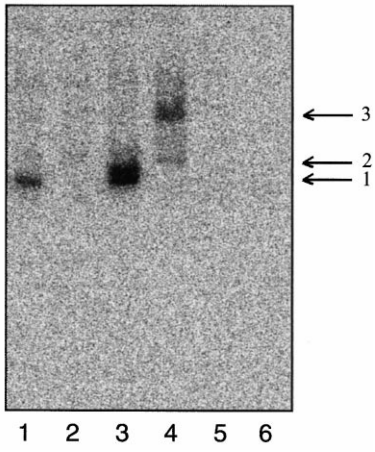

Fig. 3. Incorporation of ^{63}Ni by mutants with lesions in structural and maturation genes. S100 extracts (70 μg of total protein) from cells grown anaerobically in the presence of 150 nM ^{63}Ni (specific activity 190 μCi/μl) were subjected to non-denaturing PAGE and autoradiographed. MC4100 (lane 1), HD705 (Δ*hycE*) (lane 2), HD707 (Δ*hycG*) (lane 3), HD709 (Δ*hycI*) (lane 4), DHP-C (Δ*hypC*) (lane 5), DHP-D (Δ*hypD*) (lane 6). Radioactive materials in lane 2 may represent the large subunit of hydrogenase 1 or 2. Characteristic bands are marked by arrows.

against formate–hydrogenlyase components reacted with it (data not shown). Conversely, in a Δ*hycG* strain, HycE was solely found in the S100 and it was processed (Fig. 1A,B, lane 3). The conclusion is that processing of HycE can take place in the absence of HycG and that association with the membrane can only be seen when both large and small subunits were formed. As already reported, the resulting mature subunit does not exhibit any hydrogenase activity [22]. Intriguingly, HycG was in the free state in extracts from mutants with lesions in *hycI*, the endopeptidase encoding gene (Fig. 1C,D, lane 4), and with lesions in *hypC*, the chaperone-type protein encoding gene (Fig. 1C,D, lane 5). In these cases, as expected, HycE was unprocessed (Fig. 1B) and, as previously shown [11], migrated essentially as a multitude of conformers (Fig. 1A). The additional faster migrating species in the HD709 extract corresponds to the HypC–pre-HycE complex [14]. Immunoreactive material was only present in the S100 fraction. The results support the notion that the maturation intermediates of the large subunit are soluble and are not associated with the small subunit HycG.

3.2. Effect of nickel incorporation on the cellular distribution of HycE and HycG

Previous experiments with different biological systems had shown that growth in the absence of nickel in the medium leads to the accumulation of unprocessed large subunit. In the case of *Azotobacter vinelandii* [28] and *E. coli* [25], this precursor could be processed to the mature size when nickel was added in vitro to extracts from such cells. Fig. 2 displays the results of an analysis of the cellular distribution of HycE and HycG in strain HYD723 (*nik*$^-$) [2], which has a lesion in the nickel uptake system, grown in absence or in presence of nickel. Cell extracts from MC4100 and HD705 (Δ*hycE*) were included as controls. HYD723 grown in the absence of nickel (Fig. 2, lane 3) contained HycE and HycG solely in the soluble fraction. HycE was in the precursor form (Fig. 2B) and migrated as many conformers upon non-denaturing PAGE (Fig. 2A). HycG migration was in the position of the free form (Fig. 2C, lanes 2 and 3). When cultivation was in the presence of 400 μM nickel, HycE was essentially processed (Fig. 2B). At least half of the HycE and HycG became membrane-associated and migrated as a complex in the non-denaturing gel (Fig. 2A,C, lane 4). Hence, addition of nickel allows (i) completion of the maturation process by the C-terminal cleavage of the large subunit, (ii) association of the newly formed processed HycE with the small subunit and (iii) anchoring of the HycE–HycG complex to the membrane.

3.3. The large subunit matured in the absence of the small subunit contains nickel

The results reported had shown that the maturation process of the large subunit as visualized by C-terminal processing can occur in the soluble state and in the absence of the small subunit. The nickel incorporation experiment, the results of which are given in Fig. 3, demonstrates that it also contains nickel (Fig. 3, lane 3). The band associated with ^{63}Ni (band 1) migrates in the same position as free HycE from S100 extracts of MC4100 (Fig. 3, lane 1). When HD709 (Δ*hycI*) which is devoid of the endopeptidase was analyzed, two major ^{63}Ni-labelled pre-HycE bands appeared, one of them being associated with HypC (band 2) [14]. The other band corresponds to a multitude of conformers of pre-HycE (see below). Two other mutants with lesions in the maturation pathway, DHP-C (Δ*hypC*) and DHP-D (Δ*hypD*), were also analyzed and nickel incorporation could not be detected under these experimental conditions.

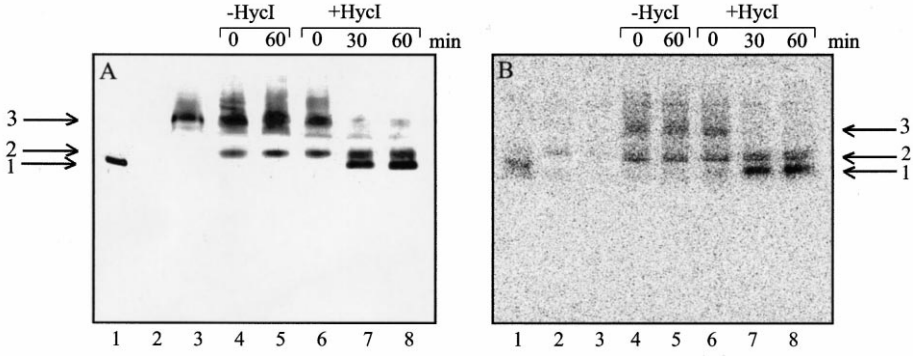

Fig. 4. In vitro processing of pre-HycE from a HD709 (Δ*hycI*) strain extract. A: Immunoblot using anti-HycE antibodies; B: Autoradiogram of ^{63}Ni. 70 μg of total protein from S100 extracts was applied on each lane. MC4100 (lane 1), HD705 (Δ*hycE*) (lane 2), DHP-C (Δ*hypC*) (lane 3), HD709 (Δ*hycI*) (lanes 4–8). Characteristic bands were marked by arrows.

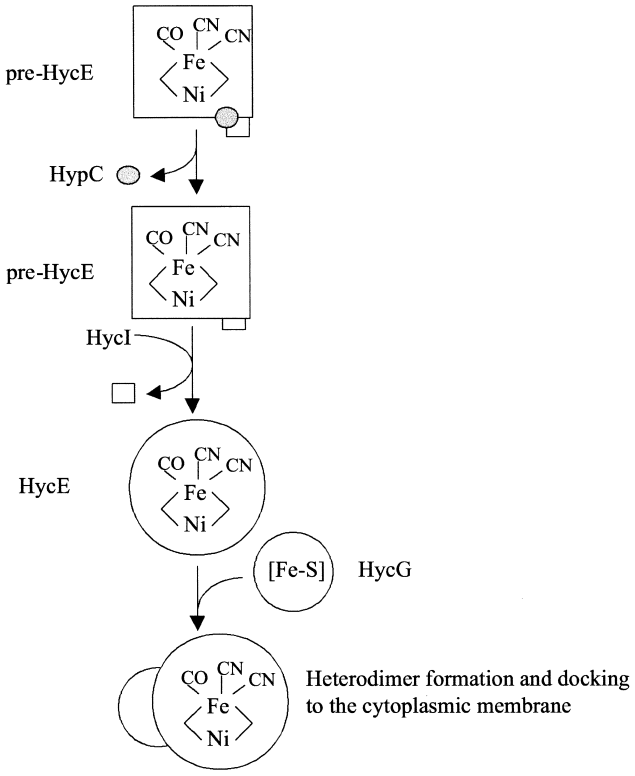

Fig. 5. Model of maturation of the hydrogenase 3 from *E. coli*.

3.4. The HypC-free pre-HycE is the substrate for the endopeptidase

The appearance of two major nickel-containing HycE precursor forms in the HD709 strain (devoid of endopeptidase activity) from which one of the conformers solely forms a complex with HypC prompted the analysis on whether the endopeptidase acts on the complex or on the free form. To this end, cultures of HD709 were grown in the presence of ^{63}Ni, S100 extracts were prepared and subjected to in vitro processing by the addition of purified endopeptidase (Fig. 4). The processing was monitored by PAGE under non-denaturing conditions followed by immunoblotting using antiserum directed against HycE (Fig. 4A) and by autoradiography (Fig. 4B). As noted before [11], processing in vitro occurs as documented by a dramatic shift of the migration of HycE under non-denaturing PAGE. Intriguingly, band 2 which represents the HypC–pre-HycE complex did not change in its intensity (Fig. 4A). This result is corroborated by those from the nickel incorporation experiment (Fig. 4B). First, the radioactivity associated with the HypC–pre-HycE complex does not change during maturation and second, there is a direct precursor product relation during processing between the material in band 3 (and other conformers) and newly generated material of band 1 which represents processed free HycE.

In conclusion, maturation of the large subunit of hydrogenase 3 as depicted in the model in Fig. 5 can take place in the absence of the small subunit; docking to the membrane, however, requires both subunits. The results presented bear relevance for the maturation of hydrogenases in those organisms which translocate the enzyme to the periplasmic space. This export is directed by the signal peptide of the small subunit which possesses the twin-arginine motif [29–33]. When this signal peptide is removed, the enzyme stays soluble and is catalytically active [31–33]. Maturation of the large subunit of periplasmic hydrogenases must take place therefore within the cytoplasm but it is open whether it occurs at the free large subunit and is followed by heterodimer formation like in the case of hydrogenase 3 or vice versa.

Our studies also showed that C-terminal endoproteolytic cleavage of the HycE precursor depends on the prior resolution of the HypC–pre-HycE complex. One of several possible speculations on the function of HypC in this complex could be to prevent association with the small subunit in order to keep the metal binding sites accessible to the endopeptidase. In the heterodimer, the metal center would be shielded since it is located close to the interface between large and small subunit [17]. It will be important now to identify conditions and components which are involved in HypC–pre-HycE dissociation.

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