Fidelity of metal insertion into hydrogenases

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Abstract The fidelity of metal incorporation into the active center of hydrogenase 3 from *Escherichia coli* was studied by analyzing the inhibition of the maturation pathway by zinc and other transition metals. Hydrogenase maturation of wild-type cells was significantly affected only by concentrations of zinc or cadmium higher than 200 μM, whereas a mutant with a lesion in the nickel uptake system displayed a total blockade of the proteolytic processing of the precursor form into the mature form of the large subunit after growth in the presence of 10 μM Zn^{2+}. The precursor could not be processed in vitro by the maturation endopeptidase even in the presence of an excess of nickel ions. Evidence is presented that zinc does not interfere with the incorporation of iron into the metal center. Precursor of the large subunit accumulated in nickel proficient cells formed a transient substrate complex with the cognate endoprotease HycI whereas that of zinc-supplemented cells did not. The results show that zinc can intrude the nickel-dependent maturation pathway only when nickel uptake is blocked. Under this condition zinc appears to be incorporated at the nickel site of the large subunit and delivers a precursor not amenable to proteolytic processing since the interaction with the endoprotease is blocked. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: [NiFe] hydrogenase; Ni incorporation; Metal center; Zinc inhibition; Endopeptidase

1. Introduction

The acquisition of nickel and its insertion into nickel-dependent enzymes like urease, CO dehydrogenase or hydrogenase involve the activity of a multitude of so-called accessory proteins (for reviews see [1–3]). Of particular interest are those steps in the maturation process which guard the specificity of incorporation. As to the present state of information there are four different checkpoints which the metal has to pass to reach its final destination. The first one is the uptake into the cell by transport systems which can be of the high affinity and specific type or of the low affinity type normally responsible for the uptake of some other metal (for review see [4]). The second one, less well defined biochemically, involves the function of some shuttle system binding the metal after entry and delivering it to the final target protein, which – in step three – by some special folding aid is held in an open conformation amenable for metal insertion. The fourth step finally consists in a mechanism rendering the acquisition of the metal thermodynamically irreversible.

In the case of hydrogenases, the metal center (situated in the large subunit) is binuclear consisting of an iron and nickel atom ligated to the protein via four cysteine thiolates two of which bridge the two metals. The iron carries two cyano and one carbonyl ligand (for review see [5]). It is more or less unknown how the iron is incorporated, however, iron insertion definitely precedes that of nickel because the precursor of the large subunit which accumulates in *Azotobacter* or *Escherichia coli* strains devoid in the uptake of nickel can be matured in vitro by the sole addition of nickel [6,7].

Concerning the specificity of nickel acquisition and incorporation into hydrogenases from *E. coli*, a specific ABC transporter has been identified [4] and a putative nickel shuttle protein, designated HypB, has been characterized which can bind nickel or – in some organisms – even store nickel [8,9]. HypB is a GTPase and it is speculated that GTP hydrolysis is involved in the donation of the metal to the hydrogenase large subunit [10]. A chaperone-like protein, HypC, was shown to form a complex with the precursor of the large subunit of hydrogenase 3, and is thought to generate the open conformation required for nickel addition [11,12]. Finally, once nickel has been transferred, an endopeptidase proteolytically removes a C-terminal extension from the large subunit [6,13,14] triggering a conformational switch which internalizes the metal center [11,15]. It was shown that the endopeptidase uses the nickel of the precursor of the large subunit as a recognition motif and it has been postulated that the endopeptidase forms a substrate complex with the nickel-containing large subunit by interacting with the metal [16,17].

To further characterize the steps involved in the fidelity control of nickel insertion we have studied the competition by other transition metals, mainly zinc, both in vitro and in vivo.

2. Materials and methods

2.1. Strains and growth conditions

E. coli NCI900 (MC4100, *nik*, Δ*hycI*) [7] cells were grown anaerobically at 37°C in a buffered rich medium (TGYEP) as previously described [11]. Sodium molybdate and sodium selenite were present at 1 μM each. Nickel-limited medium contained 50 μM EDTA in addition. The transition metals $FeSO_4$, $CdCl_2$ or $ZnCl_2$ were added to the medium at the indicated concentrations. When required, ampicillin (15 μg ml^{-1}) or chloramphenicol (30 μg ml^{-1}) were included in the growth medium. The cells were harvested after reaching an A_{600} of 1.0 and stored at −20°C.

2.2. In vitro hydrogenase processing assay

Crude extracts were prepared as described previously [11] with the

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exception that all the procedures were performed under strictly anaerobic conditions in a glove box (95% N_2, 5% H_2). They were subjected to the in vitro processing assay [7] by mixing with different components as described and further incubated at 37°C. The reaction was stopped by addition of the sample buffer and by immediate transfer to 0°C.

2.3. Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Proteins were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels or on 10% non-denaturing gels as specified previously [11]. An amount of 40 μg of total protein was applied per lane. After electrophoretic separation, the proteins were transferred onto nitrocellulose membranes and the membranes were reacted with antibodies (1:1000 dilution) raised against the large subunit of the hydrogenase 3 (HycE) and the endoprotease (HycI).

3. Results and discussion

Employing the hydrogenase in vitro maturation system of *Azotobacter* it has been shown previously that zinc when applied at a considerable concentration (400 μM) is a potent competitor and thereby inhibitor of the maturation reaction, as judged by the conversion of the precursor into the mature (i.e. processed) form of the large subunit [6]. Prompted by this strong inhibitory capacity it was tested whether zinc could interfere with in vivo maturation of *E. coli* MC4100 hydrogenase 3. No effect was seen when $ZnCl_2$ was included into the medium (TGYEP) at concentrations below 200 μM when the medium was not fortified with additional nickel. At higher concentrations, proteolytic processing of the large subunit was partially inhibited. In the presence of 800 μM zinc about 70% of the large subunit (HycE) was present in the precursor form (preHycE) (data not shown). This resistance to inhibition of wild-type cells in vivo can be interpreted in terms of a shutdown of the cellular uptake of zinc or the induction of zinc tolerance systems which prevent the build-up of effective concentrations in the cytoplasm.

To circumvent such a problem, strain NCI900 was used which carries a defect in the nickel uptake (*nik*) system plus a lesion in *hycI* which encodes the maturation endopeptidase [7]. When NCI900 cells were grown in a medium devoid of nickel the large subunit of hydrogenase 3 was present solely in the precursor form; it could be matured to about 50% upon in vitro addition of nickel plus purified endopeptidase HycI (Fig. 1, lanes 1–5). When cultivation took place in the presence of 100 μM zinc, the precursor could not be processed any more (lanes 6–10), even when 400 μM nickel was offered at the same time. Growth in the presence of cadmium delivered

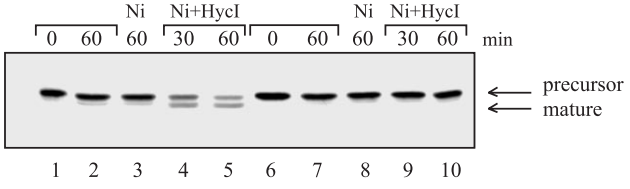

Fig. 1. Immunoblot analysis of the precursor and mature forms of HycE in extracts from nickel-limited cells and Zn^{2+}-supplemented cells after in vitro processing. Crude extracts (40 μg of total protein) were subjected to in vitro processing, separated by SDS–PAGE and reacted with antibodies raised against HycE. The following extracts were applied onto the gel: NC1900 cells grown in presence of 50 μM of EDTA (lanes 1–5) and NC1900 cells grown in presence of 100 μM of $ZnCl_2$ (lanes 6–10). The in vitro processing assays were performed under strictly anaerobic conditions as described in Section 2 using 400 μM of $NiCl_2$.

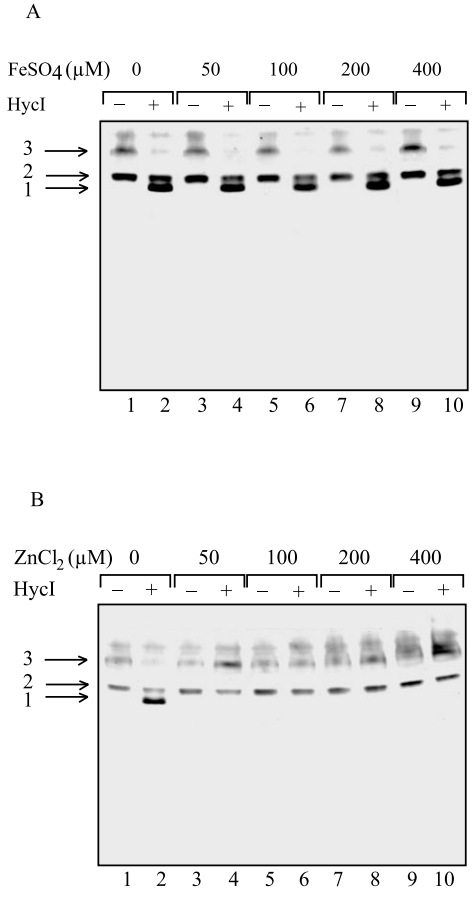

Fig. 2. Competition between nickel and other transition metals in the in vitro processing of preHycE from nickel-limited cells. Crude extracts (40 μg of total protein) from NCI900 cells grown in presence of 50 μM of EDTA that had been subjected to in vitro processing assay were separated on a non-denaturing PAGE and reacted with antibodies raised against HycE. A: Effect of Fe^{2+}. B: Effect of Zn^{2+}. $NiCl_2$ was added at a final concentration of 100 μM and the reaction mixtures were all incubated for 1 h. Band designation: processed HycE (band 1), HypC–preHycE complex (band 2) and preHycE (band 3).

identical results, whereas inclusion of Fe^{2+} in the medium did not influence in vitro processing (data not shown).

To assess competition between nickel and Fe^{2+} and Zn^{2+} more directly, in vitro processing reactions employing purified HycI endopeptidase were carried out at different metal stoichiometries (Fig. 2). Non-denaturing PAGE was used in order to assess more precisely the nature of the processed (band 1) and unprocessed forms (bands 2 and 3) of HycE. As previously reported, a band corresponding to the HypC–preHycE complex (band 2) can be distinguished among the unprocessed forms of HycE under such experimental conditions [11,12]. It was found that the presence of up to 400 μM of Fe^{2+} did not disturb the nickel-dependent processing of the hydrogenase 3 (Fig. 2A). Addition of $ZnCl_2$, in contrast, strongly interfered with it (Fig. 2B). Presence of 50 μM of $ZnCl_2$ was sufficient to fully compete with 100 μM of $NiCl_2$ in the assay and to block processing (Fig. 2B, lane 4). Interestingly, presence of $ZnCl_2$ did not significantly alter the formation of a complex between HypC and preHycE (Fig. 2B, band 2) although nickel-dependent processing was completely abolished. In a complementing experiment it was shown that 15 μM Zn^{2+} was sufficient to completely prevent processing of a precursor of the large

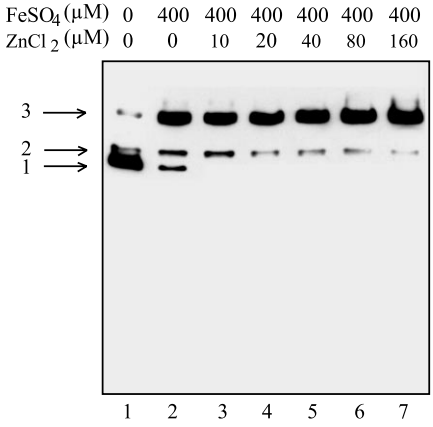

Fig. 3. In vivo competition between different transition metals on their ability to interfere with the in vitro processing of preHycE. Crude extracts from NCI900 cells grown in presence of 400 μM $FeSO_4$ and increasing $ZnCl_2$ concentrations (from 10 μM to 160 μM) have been subjected to in vitro processing assay in presence of both HycI and 400 μM of $NiCl_2$. The samples were separated by non-denaturing PAGE and reacted with antibodies raised against HycE. The following extracts were applied onto the gel: NCI900 grown with 50 μM EDTA (lane 1), 400 μM $FeSO_4$ (lane 2), 400 μM $FeSO_4$ and 10 μM $ZnCl_2$ (lane 3), 400 μM $FeSO_4$ and 20 μM $ZnCl_2$ (lane 4), 400 μM $FeSO_4$ and 40 μM $ZnCl_2$ (lane 5), 400 μM $FeSO_4$ and 80 μM $ZnCl_2$ (lane 6) and 400 μM $FeSO_4$ and 160 μM $ZnCl_2$ (lane 7). Band designation: processed HycE (band 1), HypC–preHycE complex (band 2) and preHycE (band 3).

subunit devoid of nickel and that a 50-fold excess of nickel (800 μM) only partially reverted inhibition (data not shown).

A plausible assumption derived from the results presented is that zinc binds to the nickel site during maturation of the large subunit and that the incorporation is irreversible. They leave open, however, whether there is also competition with iron incorporation. To follow this possibility it was tested whether high concentrations of iron in the medium can protect the precursor of the large subunit against the inhibitory action of zinc in the processing reaction (Fig. 3). To this end, NCI900 cells were cultured in the presence of 400 μM $FeSO_4$ plus different concentrations of $ZnCl_2$ (see top of Fig. 3) and extracts were reacted with nickel and purified endoprotease HycI [18], separated by non-denaturing PAGE and subjected to immunoblotting using antibodies against the large hydrogenase subunit. The results show that iron does not protect against inhibition of processing by zinc.

Incorporation of a false metal could have either one of two consequences: a precursor of the large subunit containing the wrong metal may not be bound by the endopeptidase since the recognition motif is lacking. Alternatively, binding of the endopeptidase takes place but it is unproductive, i.e. does not lead to cleavage of the precursor. In the latter case, a complex between the endopeptidase and the precursor loaded with the wrong metal should be expected.

To follow this issue, strain NCI900 was grown in medium devoid of nickel in the presence and absence of zinc or iron. Extracts of the cells were challenged with nickel plus purified endopeptidase HycI and analyzed by non-denaturing gel electrophoresis followed by immunodetection with the aid of antisera directed against HycE or HycI. As shown before, addition of both nickel and HycI to extracts of cells grown in the absence of nickel or zinc allows substantial processing of preHycE as visualized by the appearance of a fast migrating species (Fig. 4A, lanes 4 and 5, band 1). The extent of processing obtained with extracts from Fe-supplemented cells (Fig. 4A, lanes 9 and 10, band 1) was in the order of the extent observed with extracts from nickel-limited cells. In contrast, no processed HycE could be obtained when crude extracts from zinc-supplemented cells were used (data not shown).

When an immunoblot analysis using antibodies directed against HycI was carried out, three different bands were re-

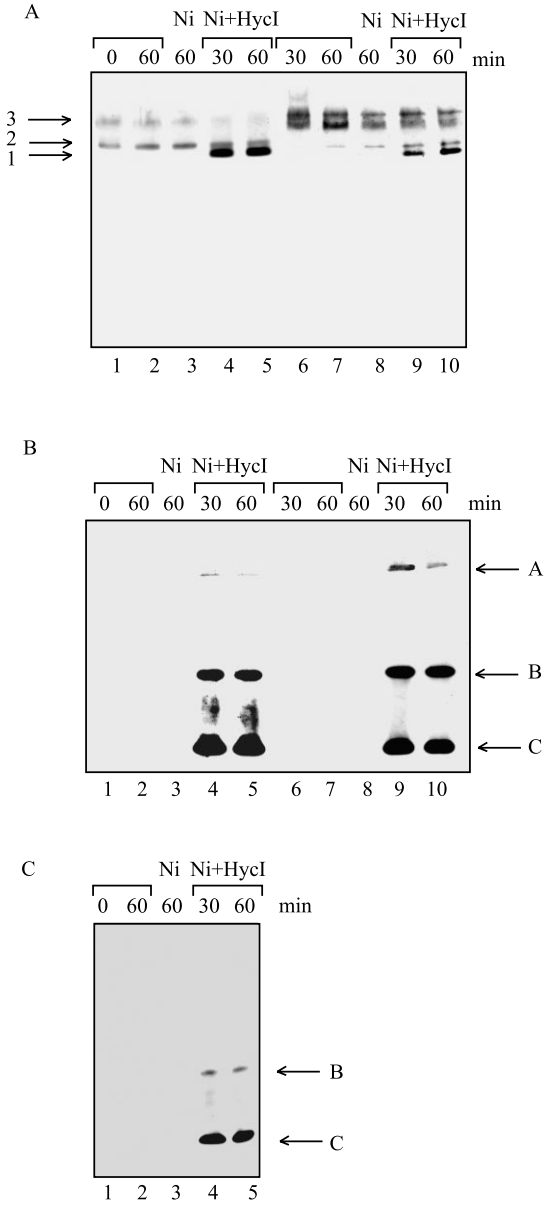

Fig. 4. Immunoblot analysis of HycE maturation intermediates in extracts from nickel-limited cells, Fe^{2+}-supplemented cells and Zn^{2+}-supplemented cells after in vitro processing. Crude extracts (40 μg of total protein) were subjected to non-denaturing PAGE and reacted with antibodies raised against HycE (A) or against HycI (B and C). The following extracts were applied on the gel: NCI900 cells grown in presence of 50 μM of EDTA (A and B, lanes 1–5). NCI900 cells grown in presence of 100 μM of $FeSO_4$ (A and B, lanes 6–10) and NCI900 cells grown in presence of 100 μM of $ZnCl_2$ (C, lanes 1–5). $NiCl_2$ was added at a final concentration of 400 μM. Band A represents the HycI–preHycE complex, band B and C represent different conformers of HycI.

vealed (Fig. 4B,C). Bands B and C represent different conformers of HycI which naturally exist in crude extracts of wild-type cells (data not shown); the fastest migrating species most likely is free and monomeric HycI. An additional band is present in crude extracts prepared from both nickel-limited cells (Fig. 4B, lanes 4 and 5, band A) and from Fe-supplemented cells (Fig. 4B, lanes 9 and 10). This band A material is absent in extracts from Zn^{2+}-supplemented cells (Fig. 4C, lanes 4 and 5). Band A reacts with anti-HycE and anti-HycI sera when immunoblot analysis using both antisera was carried out (data not shown). Therefore, band A represents a complex between the endopeptidase HycI and the nickel-containing preHycE. The complex is transient as it disappears during the time course of the assay; thus it has all the properties of an endopeptidase–substrate complex which cannot be formed when nickel in the large subunit is replaced by zinc.

In conclusion, fidelity of nickel incorporation into the large subunit depends on the existence of an active uptake system which delivers nickel to the intracellular maturation machinery. Knock-out of nickel import allows intrusion of other metals like zinc into the nickel insertion machinery. It is yet unknown at which hydrogenase maturation step zinc intrudes but the results presented suggest that in the absence of nickel delivery zinc can bind to the nickel site in the large subunit irreversibly and that the resulting zinc-containing precursor does not enter a substrate complex with the endopeptidase. It is known from the X-ray structure of HybD, which is the endopeptidase specific for the large subunit of hydrogenase 2 from *E. coli*, that three amino acid side chains are involved in the binding of a cadmium ion from the crystallization buffer [17]. Replacement of these residues interfered with proteolytic processing [16]. Direct binding of the endopeptidase to the nickel-loaded precursor demonstrated here now proves that nickel is an essential recognition motif for the endopeptidase. It also emphasizes that the processing reaction is important for controlling the fidelity of the incorporation process since a precursor containing Zn^{2+} is not bound by the endopeptidase and possibly diverted into the degradation pathway.

An issue still open concerns the role of the HypB protein in metal discrimination. Defects in HypB function can be complemented chemically by inclusion of high nickel concentrations in the medium [19]. So it is plausible to assume that apart from a nickel donor role HypB may also have a function in differentiating the correct from false metals. Information on such a role requires further experimentation.

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