Analysis of the HypC-HycE Complex, a Key Intermediate in the Assembly of the Metal Center of the Escherichia coli Hydrogenase 3*

The formation of a complex between the specific chaperone-type protein HypC and the precursor form of the large subunit HycE in the maturation pathway of hydrogenase 3 from Escherichia coli has been studied by targeted replacement of amino acids in both proteins. HypC and its homologs contain the motif MC(L/I/V)(G/A)(L/J/V)P at the amino terminus, from which the methionine residue is post-translationally removed. The exchange of the cysteine residue led to complete loss of the ability to interact with the precursor form of HycE, but replacement of the proline residue had no effect. Site-directed replacement of the conserved cysteine residues in HycE involved in nickel binding was also performed. Exchange of Cys241 resulted in the inability of the HycE variant to interact with HypC and to incorporate nickel. The variants of HycE in which Cys244 and Cys531 were replaced by alanine residues were unable to incorporate nickel, although the mutated proteins could interact with HypC. Intriguingly, the precursor of HycE in which the Cys534 residue was exchanged could form the complex with HypC, could incorporate nickel, and was C-terminally processed, but it delivered an inactive enzyme. Our findings are in favor of a model in which binding of HypC masks Cys241; Cys 244 and Cys 531 bind the iron and nickel moieties, respectively; and C534 closes the bridge between the two metals after C-terminal processing has taken place.

Despite the growing knowledge about the structures and catalytic or other functional roles for metal centers in many metalloproteins, information as to how metal centers are biosynthetically assembled is scarce. Recent studies on several systems (1–5) revealed the involvement of a surprisingly complex cascade of reactions in the maturation process in which numerous auxiliary proteins are implicated. One class of them was found to participate in metal center assembly by stabilizing a partially unfolded apoprotein conformation that is competent for the specific binding of a metal ion or a metal cofactor (1–5). This mechanism is reminiscent of the role postulated for molecular chaperones, which facilitate the correct folding of nascent polypeptide chains in vivo but are not components of the final structures. Accordingly, these particular auxiliary proteins were designated specific chaperone-type proteins (1–5), their mode of action and the basis of their specificity being not yet fully understood.

One type of enzymes whose metal center formation has been studied in some detail are hydrogenases. They play a central role in the metabolism of many microorganisms by catalyzing the production or consumption of molecular hydrogen according to the reaction, \( \text{H}_2 \leftrightarrow 2\text{H}^+ + 2e^- \). These enzymes can be divided into two major families: iron-only hydrogenases ([Fe]-hydrogenases) and nickel-iron hydrogenases ([NiFe]-hydrogenases) (6, 7). They are composed of a small electron transfer subunit (28–35 kDa) and a large catalytic subunit (45–65 kDa). In [NiFe]-hydrogenases, the large subunit harbors the metal center ligated to the protein by one motif in the amino-terminal portion of the polypeptide (RXCX3CX2H) and a second one at the carboxyl-terminal region (DPCX3CX2(H/R)) (6, 8). A major and so far highly conserved characteristic of [NiFe]-hydrogenases is their complex biogenesis, which involves at least seven accessory gene products (9). Although this pathway is far from being understood, substantial progress has been made regarding the maturation of the catalytic subunit. A current model suggests that maturation is initiated by the binding of iron together with the diatomic ligands followed by nickel insertion. Finally, the metal-containing precursor is converted to the mature form by proteolytic removal of a C-terminal extension, mediated by a specific protease (10–13). The three-dimensional structure of one of these proteases, namely the hydrogenase 2-specific protease of Escherichia coli, HybD (14), has been resolved recently by x-ray crystallography. Another accessory protein, HypC, is a specific chaperone-type protein required for the maturation of the catalytic subunit of the hydrogenase 3 (5). Such genes coding for auxiliary proteins have been found in all organisms synthesizing [NiFe]-hydrogenases.

Here we report on the interaction between the precursor form (pre-HycE) of the large subunit of the hydrogenase 3 from E. coli and the chaperone-type protein HypC. We identified cysteines both in HypC and pre-HycE as being essential for the interaction. A model is presented for the role of the cysteine residues of pre-HycE in the metal incorporation process.

**Experimental Procedures**

Bacterial Strains—E. coli DH5α was used as host for plasmid construction and maintenance. The other strains employed in this study were derivatives of E. coli MC4100 (15), namely HD705 (MC4100, ΔhycE) (16), HD709 (MC4100, ΔhycI) (10), and DHP-C (MC4100, ΔhycC) (17).

Site-directed Mutagenesis—Site-directed mutagenesis was performed by recombinant polymerase chain reaction either on the pAce1 plasmid (18) to yield hycE mutants or on the pA1021 plasmid (17) to yield hycC mutants. The Expand High Fidelity polymerase chain reaction system (Roche Molecular Biochemicals) was used. This approach is based on the polymerase chain reaction amplification of an entire plasmid DNA by mutagenic primers divergently oriented but overlapping in their 5′-ends (19–21). The resulting linear DNA molecules are then

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**To whom correspondence should be addressed. Tel.: 89-2180-6134; Fax: 89-2180-6122; E-mail: Axel.Magalon@lrz.uni-muenchen.de.**

Axel Magalon‡ and August Böck

From the Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Strasse 1a, 80638 München, Germany

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transformed into the recA DH5α strain and replicating circles are subsequently recovered by recombination between the homologous ends of the linear template. The cysteine residues at positions 241, 244, 531, and 534 of HycE were replaced by alanine via this procedure using the following primer pairs: EC241A-EC241Arev, EC244A-EC244Arev, EC531A-EC531Arev, and EC534A-EC534Arev (Table I). The Cys2 and the Pro6 residues of HypC were replaced using the following primer pairs: HypC2rev-HypC2A, HypC2rev-HypC2S, HypC2rev-HypC2R, and HypC2rev-HypCP6 (Table I). All mutations were confirmed by DNA sequencing. The resulting mutated plasmids were used to transform E. coli HD705 in the case of the pACE1 derivatives or DHP-C in the case of the pJA1021 plasmid series.

Results

Identification of Key Residues in HypC for the Maturation of the Hydrogenase 3—Among the auxiliary proteins required for hydrogenase maturation is a family of small proteins from 80 to 110 residues in size with an acidic character (pI ∼ 4–5). They are products of hypC, hypB, or hypF genes. It was shown that HypC from E. coli is able to form a stable complex with the immature form of the large subunit of the hydrogenase 3, HycE (5). A comparison of the amino acid–derived sequence of HypC with those of homologs from other organisms is given in Fig. 1. Noteworthy is the presence of a conserved motif at the amino-terminal part of the protein, namely MC(L/V)/P(G/A)/L/V/P. Amino-terminal sequencing of the purified HypC protein indicated that the methionine residue is post-translationally removed (data not shown), yielding an N terminus with a reactive thiolate. To examine its possible role in the maturation process, this cysteine residue (Cys2) and Pro6 from HypC were chosen as candidates for site-directed mutagenesis and were replaced individually by either neutral (alanine and serine) or charged (arginine) amino acids and by alanine and threonine, respectively. The resulting pJA1021 derivatives carrying the mutated genes were used to transform strain DHP-C, which lacks the chromosomal hypC gene.

The transformants were grown under fermentative conditions, and the cells were analyzed for H2-dependent benzyl viologen reduction (Table II) and for processing of the large subunit of the hydrogenase 3, HycE (5). A comparison of the amino acid–derived sequence of HypC with those of homologs from other organisms is given in Fig. 1. Noteworthy is the presence of a conserved motif at the amino-terminal part of the protein, namely MC(L/V)/P(G/A)/L/V/P. Amino-terminal sequencing of the purified HypC protein indicated that the methionine residue is post-translationally removed (data not shown), yielding an N terminus with a reactive thiolate. To examine its possible role in the maturation process, this cysteine residue (Cys2) and Pro6 from HypC were chosen as candidates for site-directed mutagenesis and were replaced individually by either neutral (alanine and serine) or charged (arginine) amino acids and by alanine and threonine, respectively. The resulting pJA1021 derivatives carrying the mutated genes were used to transform strain DHP-C, which lacks the chromosomal hypC gene.

The transformants were grown under fermentative conditions, and the cells were analyzed for H2-dependent benzyl viologen reduction (Table II) and for processing of the large subunit HycE (Fig. 2). Transformants carrying plasmids in which Cys2 of HypC was replaced were devoid of hydrogenase subunit HycE (Fig. 2). Transformants carrying plasmids in which Cys2 of HypC was replaced were devoid of hydrogenase subunit HycE (Fig. 2). Transformants carrying plasmids in which Cys2 of HypC was replaced were devoid of hydrogenase subunit HycE (Fig. 2).
interpreted previously by the assumption that maturation follows a channeled pathway that requires the stoichiometric amount and activity of the components involved (17, 24–26). Any effect of overexpression of the hypC gene in trans on the maturation process can be ruled out (Table II). In agreement with the inability of the Cys2 mutant proteins to support the generation of hydrogenase activity, HycE is not processed in these strains (Fig. 2, lanes 5–7).

To gain further insight into the impairment of the HypC function, the ability of the HypC variants to form a complex with the precursor form of HycE during the maturation process was examined. A ND-PAGE system was employed to study in vivo complex formation between HypC and pre-HycE (5). S100 extracts from strains transformed with the different plasmids were separated by ND-PAGE, and Western blot experiments were performed using antisera directed against HypC. The results show that the Cys2 mutants were no longer able to form a complex with the immature form of HycE (Fig. 3). They migrate in the position of free HypC with the interesting exception of the C2S variant. There is no obvious explanation for its retarded migration, but it could be the consequence of some modification at the hydroxyl or at the amino group of the amino-terminal serine. Again, the variant in which Pro6 was replaced showed the same pattern as the wild-type protein (data not shown).

### Table II

| Strain       | Enzyme activity (μmol H₂ min⁻¹ mg⁻¹ total proteins) |
|--------------|-----------------------------------------------------|
| MC4100       | 0.56                                                |
| MC4100/pJA1021 | 0.52                                              |
| DHP-C        | 0.06                                                |
| DHP-C/pJA1021 | 0.18                                                |
| DHP-C/pJA1021(C2A) | 0.01                                           |
| DHP-C/pJA1021(C2S) | 0.01                                           |
| DHP-C/pJA1021(C2R) | 0.01                                           |
| DHP-C/pJA1021(P6A) | 0.17                                                |
| DHP-C/pJA1021(P6T) | 0.05                                                |
| HD705        | 0.05                                                |
| HD705/pACEc1 | 0.54                                                |
| HD705/pACEc1(C241A) | 0.05                                           |
| HD705/pACEc1(C244A) | 0.05                                           |
| HD705/pACEc1(C531A) | 0.06                                                |
| HD705/pACEc1(C534A) | 0.06                                                |

*The basal value of HD705 (ΔhycE) or of DHP-C (ΔhypC) represents the activities of hydrogenases 1 and 2.
replacement in HypC, a plausible assumption was that one of
the conserved cysteine residues in HycE may be also involved.
Hence, each of the cysteine residues present in the two nickel-
binding motifs of HycE were substituted by an alanine residue
via site-directed mutagenesis, which led to genes coding for the
C241A, C244A, C531A, and C534A mutant HycE proteins.
Under fermentative conditions, none of the mutants exhibited
hydrogenase 3 activity (Table II). These observations were
corroborated by the results of the immunoblotting analysis of
HycE. As already noted previously for other auxiliary proteins
involved in hydrogenase maturation (17, 18, 24, 26), the dele-
tion of the chromosomal hycE gene cannot be fully comple-
mented by expression of hycE in trans (Fig. 4, lane 3), possibly
because of some imbalance in stoichiometry between pre-HycE
and the components of the maturation machinery. Despite a
low processing level, the expression of hycE in trans yielded
hydrogenase 3 activity nearly identical to that of the parental
strain (Table II). With the striking exception of the C534A
mutant, none of the other mutant proteins were subjected to
HyC-mediated proteolysis (Fig. 4). Intriguingly, therefore,
replacement of Cys534 by alanine allows C-terminal processing
but prevents development of hydrogenase activity (Table II).

We then investigated whether the mutated HycE polypep-
dides, notably the C534A variant, could incorporate nickel. To
this end, cultures were grown in presence of 65Ni. S100 extracts
were separated by ND-PAGE, and 65Ni-labeled proteins were
identified by autoradiography (Fig. 5) and Western blot analy-
sis (Fig. 6). In the case of the parental strain MC4100 (Fig. 5,
lane 1, band 1), a major 65Ni signal comigrated with material
immunoreacting with antisem directed against HycE and
 corresponds to the mature form of HycE as expressed by ND-
PAGE (Fig. 6A, lane 1). In addition, a slower migrating 65Ni-
labeled protein band also present in the HD705 extract (which
is devoid of hycE gene) can be seen (Fig. 5, lane 2). It may
represent the large subunit of either hydrogenase 1 or 2.
In the case of the HD709 extract, two major pre-HycE conformers
produced in absence of the HycI protease and identified by
Western blot analysis (Fig. 6A, lane 3) contained 65Ni (Fig. 5,
lane 3, bands 2 and 3), one of them being associated with HycP
(band 2) (see Fig. 6). Finally, from the four HycE variants, only
that carrying the C534A replacement gave a 65Ni-labeled pro-
tein band that immunoreacted with anti-HycE antisem and
was equivalent to the signal obtained with the transformant
carrying the plasmid with the wild-type hycE gene (Fig. 5,
lanes 5 and 9, band 1). These results support the notion that
nickel incorporation in the C534A variant is not accompanied
by the generation of hydrogenase 3 activity.

In order to check if any of the cysteine substitutions in HycE
impaired the ability for complex formation with HycP during
the maturation process, each of the transformants was ana-
lyzed with the procedure described previously (5). As expected,
the C534A protein that underwent HycI-mediated processing
was able to specifically interact with HycP (Fig. 6B, lane 7).
The same result was obtained for the transformants possessing
the HycE variants C244A and C531A. On the other hand, the
C241A variant was unable to interact with HycP (Fig. 6B). It
was previously pointed out (5) that only a certain conformer of
pre-HycE is competent to bind HypC (Fig. 6A, lane 3, band 2),
whereas the majority of the immature polypeptide cannot par-
cipate in this interaction (Fig. 6A, lane 3, band 3), at least
under the experimental conditions employed. Indeed, the ab-

ence of the characteristic fastest migrating immunoreactive
form of HycE in the C241A mutant (Fig. 6A, lane 4, band 2) is
paralleled by the absence of a HypC-pre-HycE complex (Fig.
6B, lane 4).

Effect of Reducing Agents and Alkylating Agents on the Sta-
Bility of the HypC-Pre-HycE Complex—The results described
thus far had indicated that cysteine residues both in HypC and
HycE are essential for the complex formation between the two
proteins. This prompted the analysis of whether the formation
of a disulfide bridge could be responsible for the protein-protein
interaction. For this purpose, S100 extracts of strain HD709,
which contain large amount of the complex (Fig. 6B, lane 3),
were analyzed under both reducing and nonreducing conditions
by SDS-PAGE followed by immunoblotting analysis using an-
tiserum directed against HycE. There was no alteration of the
migration of the HycE polypeptide under nonreducing condi-
tions (Fig. 7, lanes 2 and 5). As a control, extracts from strain
DHP-C (ΔhycP), which contain the precursor form of HycE,
were analyzed under identical conditions, and an identical
pattern was observed. These observations provide circumstan-
tial evidence that HypC does not interact with pre-HycE via the
formation of a disulfide bridge that under nonreducing condi-
tions should give rise to a slower migrating species.

To further characterize the interaction, HD709 extracts were
treated with different alkylating or reducing reagents and then
separated by ND-PAGE. Although some dissociation occurred
as indicated by the appearance of free HypC, the presence of 10
or 25 m M dithiothreitol was not sufficient to fully resolve the
complex (data not shown). In the absence of reducing agents,
the complex is extremely stable, since no dissociation is detect-
able after 2 h of incubation.

The amenability of the cysteine residues within the complex
to alkylation was then determined by their reaction with iodo-
acetic acid or iodoacetamide. Thiols potentially masked in dis-
sulfide bonds under these native conditions were reduced by
preincubation with dithiothreitol, and the electrophoretic be-
havior of HypC associated or not with pre-HycE was examined
by ND-PAGE. The reaction of such S100 extracts from strain
HD709 treated with each of the alkylating agents either under
reducing or nonreducing conditions resulted in the complete
dissociation of the complex after only 5 min of incubation (Fig.

In vivo incorporation of 65Ni by transformants carrying
mutated hycE genes. S100 extracts (70 μg of total proteins) were
subjected to ND-PAGE and autoradiographed. Lane 1, MC4100; lane 2,
HD705 (ΔhycE); lane 3, HD709 (ΔhycE); lane 4, DHP-C (ΔhycP); lane 5,
HD705/pACE1; lane 6, HD705/pACE1-C241A; lane 7, HD705/pACE1-
C244A; lane 8, HD705/pACE1-C531A; lane 9, HD705/pACE1-C534A.
Characteristic bands are marked by arrows.

FIG. 4. Immunoblotting analysis of HycE precursor and ma-
ture forms in transformants expressing HycE mutant forms.
Crude extracts (30 μg of total proteins) were subjected to SDS-PAGE as
indicated in Fig. 2. Lane 1, MC4100; lane 2, HD705 (ΔhycE); lane 3,
HD705/pACE1; lane 4, HD705/pACE1-C241A; lane 5, HD705/pACE1-
C244A; lane 6, HD705/pACE1-C531A; lane 7, HD705/pACE1-C534A.
Precursor and mature forms of HycE are indicated by arrows.
Any accessible thiolate group not involved in disulfide bond formation should react and subsequently carry one negative charge after modification with iodoacetic acid. The different electrophoretic mobility of the liberated HypC revealed that alkylation of the protein had occurred.

**DISCUSSION**

A key role in the maturation process of [NiFe]-hydrogenases has been demonstrated recently for the chaperone-type protein HypC and its interaction with the HycE apoprotein of the *E. coli* hydrogenase 3 (5). This complex has been identified in each of the mutants with a lesion in hyp genes, supporting the idea that its formation may constitute an early step in the maturation process (5).

In the present work, it was shown that cysteine residues both from HypC and the precursor form from the large subunit are crucial for the interaction. Fig. 9 displays the function of the thiolates in liganding the [NiFe] cluster in the mature subunit (Fig. 9A) as delineated from the three-dimensional structure of the *Desulfovibrio gigas* enzyme (8) and compares it with the role postulated for these thiolates during the maturation process on the basis of the results reported (Fig. 9B). The proposed model implies that (i) Cys 241 is masked in pre-HycE by the interaction with HypC, (ii) Cys 244 coordinates the iron, (iii) Cys 531 coordinates the nickel, and (iv) Cys 534 provides a free thiolate attacking the nickel and iron atoms after proteolytic removal of the carboxyl terminus and thereby closes the bridge. Below we will discuss the evidence available that supports this model.

**Masking of Cys 241 by HypC**—When each of the four cysteine residues playing a role in the ligation of the [NiFe] cluster of the mature subunit was replaced by an alanine, the C241A variant was the only one that was no longer able to interact with HypC. It is assumed that the effect is specific, since an identical replacement of Cys 244 only 3 residues away allowed the interaction to take place. The well conserved Arg 239 residue by leucine also in close vicinity of Cys 241 did not prevent complex formation.

**Reactivity of the sulfhydryl groups in the HypC-pre-HycE complex with alkylating agents**—HD709 extracts that had been incubated with or without dithiothreitol were alkyalted with iodoacetic acid (IAA) or iodoacetamide (IAM) and separated on a ND-PAGE. HypC-pre-HycE complex and alkylated HypC are marked by arrows.

**Interaction between HypC and Pre-HycE**—When each of the four cysteine residues playing a role in the ligation of the [NiFe] cluster of the mature subunit was replaced by an alanine, the C241A variant was the only one that was no longer able to interact with HypC. It is assumed that the effect is specific, since an identical replacement of Cys 244 only 3 residues away allowed the interaction to take place. The well conserved Arg 239 residue by leucine also in close vicinity of Cys 241 did not prevent complex formation.

**Model of the functional role of the conserved cysteines in the HycE polypeptide**—A, in the mature large subunit according to the crystal structure of the *D. gigas* enzyme (41); B, in the precursor form of the large subunit.

**Fig. 6.** Immunoblotting analysis of HypC-pre-HycE complex formation in transformants carrying mutated hycE alleles on a plasmid. S100 extracts (40 µg of total proteins) were subjected to ND-PAGE and reacted using anti-HycE antibodies (A) or anti-HypC antibodies (B). Lane 1, MC4100; lane 2, HD709 (ΔhycI); lane 3, HD709 (ΔhycI); lane 4, HD709/pACE1-C241A; lane 5, HD709/pACE1-C244A; lane 6, HD709/pACE1-C531A; lane 7, HD709/pACE1-C534A.

**Fig. 7.** Effect of reducing or nonreducing conditions on the migration of the HypC-pre-HycE complex upon SDS-PAGE. After incubation with (lanes 1–3) or without (lanes 4–6) β-mercaptoethanol in the Laemmli sample buffer, S100 extracts (40 µg of total proteins) of different strains were separated by SDS-PAGE and reacted using antisera directed against HycE. Lanes 1 and 4, MC4100; lanes 2 and 5, HD709 (ΔhycI); lanes 3 and 6, DHP-C (ΔhypC).

**Fig. 8.** Reactivity of the sulfhydryl groups in the HypC-pre-HycE complex with alkylating agents. HD709 extracts that had been incubated with or without dithiothreitol were alkyalted with iodoacetic acid (IAA) or iodoacetamide (IAM) and separated on a ND-PAGE. HypC-pre-HycE complex and alkylated HypC are marked by arrows.

**Fig. 9.** Model of the functional role of the conserved cysteines in the HycE polypeptide. A, in the mature large subunit according to the crystal structure of the *D. gigas* enzyme (41); B, in the precursor form of the large subunit.

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2 A. Magalon and A. Böck, unpublished results.
ture of the interaction is unknown at present and requires the purification of substantial amounts of the maturation intermediate. It could consist in the interaction of each thiol as hydrogen donor with a strong acceptor group from the other partner. Alternatives would be that the thiol of Cys of HypC and Cys of HycE could be sandwiched by some metal ion, like iron or zinc. However, the in vitro addition of chelating agents as EDTA or 2,2'-dipyridyl has no influence on the complex stability (data not shown). Interestingly, once the complex is formed it cannot be titrated by a surplus of a HypC variant in which the N-terminal cysteine was replaced by an alanine or serine residue. 2 If the interaction were noncovalent and assuming that the specificity of recognition is not only determined by the two cysteine residues, this should have been the case. Disulfide bonding as a possibility can be ruled out because the complex is readily dissociated by iodoacetate or iodoacetamide and because no migration difference of the complex under SDS-PAGE was observed under oxidative compared with reducing conditions.

Coordination of the Iron by Cys and of Nickel by Cys—There are substantial number of arguments that the two metals are incorporated separately into the precursor of HypC. The most convincing ones are that the precursor formed in a strain grown in absence of nickel can be matured in vitro by the addition of nickel alone (11). This provides support for the contention that iron has already been incorporated and that nickel is inserted independently as a late step. Furthermore, the maturation endopeptidase scans the precursor for the presence of nickel by binding to the metal, which excludes the existence of the bridged cluster (14, 27). Evidence that Cys carries the iron in the precursor can only be deduced from the structure of the mature cluster (8). The direct interaction between Cys and nickel, however, has been proven in the case of [NiFe]-hydrogenase from Desulfovibrio baculatus in which the cysteine is naturally substituted by a selenocysteine whose selenium atom has been shown to directly interact with nickel (28, 29).

Cys of Pre-HycE Contains a Free Thiol—An intriguing finding was also that Cys of HycE is not essential for maturation. The C534A variant forms a complex with HypC, accepts nickel, and is proteolytically processed but does not yield an active enzyme. Incorporation of nickel and processing, although not interaction with HypC, was recently demonstrated for an analogous mutant in the large subunit of the NAD-reducing hydrogenase of Alcaligenes eutrophus (26). Cys which provides the thiolate for bridging the iron and the nickel in the mature cluster, does not interact with the metals during the maturation process.

Working Model—Based on these results, a working model on the sequence of events in the assembly of the [NiFe] center is proposed (Fig. 9). First, the thiolate of Cys is masked by interaction with HypC. Since nickel can be incorporated into this complex, Cys cannot be involved in the initial ligation of the metals of the center. Second, iron binding takes place at Cys and nickel is coordinated by Cys.

The key intermediate for the assembly of metal center, therefore, is the complex between pre-HycE carrying the iron at Cys and the chaperone-type protein HypC. It is assumed that the C terminus of HycE sticks out of the complex so that nickel can be added to Cys. By an unknown reaction, the linkage between pre-HycE and HypC is then cleaved, rendering pre-HycE a substrate for the endopeptidase. Only nickel-containing pre-HycE from which HypC has been released can be cleaved by the endopeptidase. 2 This cleavage triggers a conformational switch feeding the C terminus toward the two metals and closing the bridge between them by reaction with the free thiol of Cys.

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