reduction and volatilization of mercury, due to
the presence of the mercury-resistance determina
t Tn5073 (A. M. M. Essa, D. J. Julian, S. P.
Kidd, N. L. Brown and J. L. Hobman, unpublished work). The second mechanism is the
aerobic precipitation of ionic Hg\(^{2+}\) as insoluble
HgS, as a result of H\(_2\)S production. The third is
the biomineralization of Hg\(^{2+}\) as an insoluble
mercury-sulphur complex other than HgS. We
believe that this is due to the aerobic production of
a volatile thiol compound. This process showed
high efficiency of mercury removal in the presence
of high concentrations of mercury and at different
pH and salinity levels (A. M. M. Essa, L. E.
Macaskie and N. L. Brown, unpublished work),
and therefore may be applicable in an industrial
process with minimal prior treatment of the waste
water.

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Metal Insertion into Proteins

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Abstract
The synthesis and the insertion of the metallo-
centre of NiFe-hydrogenases is a complex pro-
cess, in which seven maturation enzymes plus
ATP, GTP and carbamoyl phosphate are in-
volved. The review summarizes what is known
about the properties and activities of these aux-
iliary proteins, and postulates a pathway along
which maturation may take place.

Introduction
The synthesis and assembly of enzymes containing
metal centres is a relatively new field in bio-
chemistry. It emerged during mutational analyses
that, in addition to mutations in the structural
genes of metalloenzymes, there are additional
genetic lesions that prevent the generation of active
enzymes. These so-called auxiliary proteins may
possess a variety of functions, some of them
hitherto unprecedented. These include specific
cellular uptake of the metal, binding to an in-
tracellular metallochaperone or shuttle system,
donation of the metal to the apoprotein and
release of the metal donor thereafter, keeping the
folding of the apoprotein in a competent form.
Other functions may reside in changing the folding
state to internalize the metal centre after com-
pletion of the incorporation, or involve the
synthesis of organic moieties to which the metal is
attached prior to incorporation into the apo-
protein, as in the case of the molybdopterine
cofactors (for reviews see [1–4]).
Auxiliary systems are limited almost exclusively to the formation of enzymes containing transition metals, such as iron, copper, molybdenum and nickel. The reason for this could be that the intracellular environment, with its high concentrations of metal-complexing chemical groups, such as thiols, imidazoles and carboxy groups, would block access of the metal to its apoprotein by pure diffusion. Also, the heavy metal could be toxic unless it is bound to some shuttle system.

An intriguing issue concerns the insertion of one and the same metal into different apoproteins to deliver metalloenzymes with completely different functions. In the case of nickel-dependent enzymes, for example, such as urease, hydrogenase and CO dehydrogenase, most of the auxiliary proteins are specific and cannot be exchanged. A few of them, however, have the same functions, such as the GTPases (ATPase in the case of CooC) involved in the maturation of all three classes of nickel-dependent enzymes mentioned above [5–7]; and in a singular instance it was demonstrated recently that two such auxiliary proteins function in both urease and hydrogenase maturation [8].

 Auxiliary proteins involved in hydrogenase maturation

NiFe-containing hydrogenases constitute by far the most complex system for the formation of a nickel-dependent enzyme. The metal centre of such hydrogenases consists of an iron atom and a nickel atom hooked to four cysteine thiolates of the protein backbone of the large subunit, two of which serve as ligands bridging the two metals (Figure 1) ([9]; for review, see [10]). In addition, the iron atom carries two cyano (CN) and one carbonyl (CO) ligand [11,12]. The synthesis of this binuclear centre, therefore, presents an intriguing and novel issue of bioinorganic chemistry.

Genes coding for products with a function in the formation and insertion of this NiFe centre were first characterized for Escherichia coli [13,14]. Analysis of other biological systems, including archaea, showed that the amino acid sequences of the gene products, and in bacteria also the chromosomal organization, are strongly conserved. Table 1 gives a summary of the complement of auxiliary proteins involved in hydrogenase maturation in E. coli, and also presents some structural characteristics of these proteins and their role in the maturation process.

The genes encoding five of these auxiliary proteins (HypA, HypB, HypC, HypD and HypE) are organized in a transcriptional unit, whereas that for HypF is part of a bicistronic unit together with a gene of unknown function [15,16]. Four of the proteins (HypB, HypD, HypE and HypF) are required for the maturation of all three functional hydrogenases in E. coli, whereas HypA and HypC possess roles mainly in hydrogenase 3 maturation; the reason is that homologues of hypA and hypC are present in the operon for hydrogenase 2 (hybF and hybG; [17]) and they exert their function in the maturation of both hydrogenases 1 and 2 [14,18,18a]. A recent study showed that another general requirement for the maturation of all three hydrogenases from E. coli is the availability of carbamoyl phosphate (CP) as an educt for the synthesis of the CN or CO ligand, or both ([19]; see below).

Finally, maturation of each of the three functional hydrogenases of E. coli requires the function of an endopeptidase, which removes a C-terminal tail from the large hydrogenase subunit once nickel has been inserted [20]. Their genes are located in the operons that also encode the structural polypeptides, which results in the co-formation of the endopeptidase with the precursor of the large subunit as the substrate.

**Figure 1**

**NiFe-hydrogenase structure**

(A) Schematic representation of the large subunit polypeptide of NiFe-hydrogenases, indicating the positions and sequences of the two motifs involved in the co-ordination of iron and nickel. The hatched part represents the C-terminal extension that is removed by the endopeptidase. (B) Model of the active site of NiFe-hydrogenases according to [10–12]. The numbering of the Cys residues corresponds to that in (A).
Table I
Properties of auxiliary proteins involved in the maturation of NiFe-hydrogenases from *E. coli*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (kDa)</th>
<th>Quaternary structure</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HypA/HybF</td>
<td>$13/12.6$</td>
<td>$\alpha_1$</td>
<td>Ni insertion</td>
<td>$[8,18a]$</td>
</tr>
<tr>
<td>HypB</td>
<td>$31.4$</td>
<td>$\alpha_2$</td>
<td>GTPase, Ni insertion</td>
<td>$[5,35]$; M. Blokesch and A. Böck, unpublished work</td>
</tr>
<tr>
<td>HypC/HybG</td>
<td>$9.6/8.7$</td>
<td>$\alpha_2$</td>
<td>Chaperone, Fe insertion</td>
<td>$[30,31]$; M. Blokesch and A. Böck, unpublished work</td>
</tr>
<tr>
<td>HypD</td>
<td>$41.2$</td>
<td>$\alpha$</td>
<td>Fe-S protein, Fe insertion</td>
<td>$[32,33]$; M. Blokesch and A. Böck, unpublished work</td>
</tr>
<tr>
<td>HypE</td>
<td>$33.6$</td>
<td>$\alpha_2$</td>
<td>CO/CN synthesis, ATPase</td>
<td>A. Bauer, A. Paschos and A. Böck, unpublished work</td>
</tr>
<tr>
<td>HypF</td>
<td>$81.9$</td>
<td>$\alpha$</td>
<td>CO/CN synthesis, CP phosphatase, ATP pyrophosphorylase</td>
<td>$[19]$; A. Paschos et al., unpublished work*</td>
</tr>
<tr>
<td>Endopeptidases</td>
<td>$21.4/17.6/16.9$</td>
<td>$\alpha$</td>
<td>C-terminal cleavage</td>
<td>$[17,20,40]$</td>
</tr>
<tr>
<td>CP synthetase (CarA/CarB)</td>
<td>$41.3/17.6$</td>
<td>$\alpha\beta$</td>
<td>Provision of CO/CN precursor</td>
<td>$[19]$</td>
</tr>
</tbody>
</table>

*A. Paschos, A. Bauer, A. Zimmermann, E. Zehelein and A. Böck, unpublished work.*
Maturation pathway

Figure 2 depicts the pathway of hydrogenase maturation in *E. coli* in a linear sequence. It needs to be emphasized that this has to be regarded as a working model, since it contains direct proof of some partial reactions side-by-side with indirect evidence or even speculation for others.

There is now proof that Fe and Ni are inserted separately. Ni insertion followed by proteolytic removal of the C-terminal extension and generation of hydrogenase activity can be accomplished *in vitro* when the precursor of the large subunit from nickel-starved cells is provided with a high nickel concentration and incubated with purified endopeptidase under anoxic conditions [21]. This forces the conclusion that such a precursor must contain the iron plus CO/CN ligands. Secondly, the endopeptidase uses nickel as a recognition motif for binding to the precursor of the large subunit [22,23]. Such a complex can be detected [24]; physically, this is only possible when the Fe and Ni centres have not yet been bridged. In the following sections, detailed descriptions of the separate steps in the maturation pathway are summarized.

Synthesis of the CO/CN ligands

Although final chemical proof is still lacking, it is now well established that CP is the educt of at least one, if not both, of these ligands [19]. Evidence is as follows. (i) Mutants of *E. coli* devoid of CP synthetase activity are blocked in hydrogenase maturation; this blockade can be released by citrulline, which is an alternative source of CP [19]. (ii) The auxiliary protein HypF possesses CP phosphatase activity. Mutations in hypF that abolish this activity also prevent hydrogenase maturation (A. Paschos, A. Bauer, A. Zimmermann, E. Zehelein and A. Böck, unpublished work). (iii) Chemical model reactions are known which prove that a carbamoyl moiety can be converted into either a cyano or a carbonyl moiety [19].

CP cleavage by HypF correlates with the observation that the protein contains a strongly conserved sequence motif indicating acylphosphatase activity [25]. In addition, a signature sequence is present that is also shared by enzymes involved in O-carbamoylation reactions [19,26,27]. Finally, HypF catalyses a PPγ-ATP exchange reaction that is dependent on CP (A. Paschos, A. Bauer, A. Zimmermann, E. Zehelein and A. Böck, unpublished work). Such an exchange reaction implies the formation of an 'activated' form of CP as an intermediate. The chemical nature and biochemical function of this intermediate remain to be resolved.

It was discovered recently that HypF forms a tight complex with another auxiliary protein, HypE ([28]; A. Bauer, A. Paschos and A. Böck, unpublished work). Intriguingly, HypE carries sequence motifs that are also present in the PurM protein, which catalyses a dehydration reaction.

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*Figure 2*

**Postulated pathway of the maturation of hydrogenase 3 from *E. coli***

For details, see the text.
in purine biosynthesis involving a protein-bound phosphorylated intermediate [29]. Purified HypE indeed cleaved ATP into ADP and P_i, but the reaction velocity was not changed when CP was added (A. Bauer, A. Paschos and A. Böck, unpublished work). Again, the biochemical role of the interaction with ATP needs to be clarified, not of isolated HypE but in its complex with HypF. Although not proven, it is most probable that the two ATP-requiring reactions are crucial in the mechanism by which CP is converted into the CO and/or CN adducts.

The HypC–HypD complex

Analysis of cellular extracts from a carAB strain (devoid of CP synthetase activity) by non-denaturing PAGE led to the discovery of a complex between the auxiliary proteins HypC and HypD. This complex was immediately resolved when the cells were challenged with citrulline as a CP source, thus proving its role as an intermediate in hydrogenase maturation; the resolution was paralleled by the build-up of processed large subunit (M. Blokesch and A. Böck, unpublished work). When the ΔcarAB mutation was combined with a deletion of the gene for the large subunit of hydrogenase 3 (hycE), the addition of citrulline did not result in removal of HypC from the HypC–HypD complex. Rather, a second form of the complex developed that migrated more slowly in the non-denaturing gel (HypC*-HypD*).

HypC has been described previously as also entering a complex with the precursor of the large hydrogenase subunit. This complex was observed mainly under conditions whereby maturation could not proceed because of, for example, nickel deficiency or a mutation in the gene for the endopeptidase [30]. A chaperone-like function has thus been attributed to the protein [31]. Discovery of the existence of the HypC–HypD complex in the absence of available CP, and of the HypC*-HypD* complex in the presence of CP and its resolution when the precursor of the large subunit is present, argues for the contention that HypC also has a transfer function. It is speculated that liganding of the Fe takes place at the HypC–HypD complex, yielding the HypC*-HypD* complex, which then – in the function of a metallochaperone – transfers the liganded iron to the precursor of the large subunit. Circumstantial evidence supporting this speculation comes from experiments showing that HypC cannot enter a complex with HypD in iron-limited medium (S. Huth and A. Böck, unpublished work). Moreover, liganding of the iron with each of the three ligands requires two electrons; these could be provided by the HypD protein, which contains a 3Fe–4S or a 4Fe–4S centre [32,33].

Nickel insertion

Two auxiliary proteins, HypA and HypB, have been implicated in the insertion of nickel. The main evidence for this comes from in vivo studies with hypB [34] and hypA ([8]; M. Hube, M. Blokesch and A. Böck, unpublished work) mutants. The defects of these mutants in the maturation of hydrogenases can be partially restored by challenging the cells with high nickel concentrations. Whereas no biochemical activity has yet been attributed to HypA, HypB has been characterized as a GTPase, and it was shown that GTP hydrolysis is indeed required for hydrogenase maturation [5,35]. This holds for HypB from E. coli in hydrogenase maturation, as well as for UreG in urease maturation [6]. In the maturation of CO dehydrogenase, CooC couples ATP hydrolysis with nickel insertion into apo-CO dehydrogenase [7]. The actual requirement for GTP hydrolysis is not yet clear, although speculation on a role in metal release to the apoprotein or in dissociation of HypB from the apoprotein after nickel release has been put forward [5,14].

Binding of nickel by HypB has been demonstrated for the protein from Bradyrhizobium japonicum [36]. In this organism, HypB appears to possess a dual function: in nickel storage (accomplished by a polyhistidine stretch of the protein) and in nickel insertion. The residues of the protein involved in the latter function have not been characterized. It is also unknown why the binding constant of the purified proteins for nickel as a ligand is several orders of magnitude weaker than one would expect from the in vivo situation.

C-terminal proteolytic cleavage of the precursor of the large subunit

When both metals have been co-ordinated to the precursor of the large subunit, it is accessible to the endoproteolytic removal of the C-terminal extension by the specific endopeptidase. Proteolytic attack occurs only at the precursor from which HypC has been dissociated [37]. Since the chemical nature of the interaction between HypC and the large subunit is unknown, it is too early to draw any conclusions on the mechanism. The most plausible mechanism could consist of the withdrawal of HypC by free HypD; indeed, when HypD is overproduced in a cell, the amount of the HypC–HycE complex is decreased to low
levels (A. Magalon, and A. Böck, unpublished work).

Considerable information on the nature of the cleavage reaction has been obtained from the determination of the crystal structure of the endopeptidase HybD, which is involved in the maturation of the precursor of the large subunit of hydrogenase 2 from E. coli [22]. HybD was crystallized with a Cd$^{2+}$ ion from the crystallization buffer, and it is thought that the nickel takes the position of the cadmium when HybD interacts with its cognate substrate. The metal is pentacoordinated, involving Glu, Asp and His residues and a water molecule [22]. Mutational replacement of the respective amino acid residues Asp, Asp and His by chemically different ones in the protease of hydrogenase 3 (HycI) abolished its activity, whereas replacement by chemically similar residues caused a decrease in activity, thus proving their involvement in metal binding [23]. Purified endopeptidase is metal-free [23] and its activity is not affected by metal chelators when the substrate, i.e. the precursor of the large subunit, contains nickel. The metal thus serves as a recognition motif for the endopeptidase, and in this way also fulfills the role of a fidelity control element. It is an open question as to whether, in addition to this recognition function, the metal also participates in the mechanism of the reaction.

Considerable effort has been put into the characterization of cleavage site specificity. Many replacements of the pre- and post-cleavage site residues have been conducted, and the C-terminal extensions have been either mutagenized or successively truncated ([38,39]; E. Theodoratou and A. Böck, unpublished work). The general messages gained are: (i) although the cleavage-site residues are strongly conserved in different large subunits from both bacteria and archaea, they tolerate a surprisingly large number of exchanges without loss of substrate function; and (ii) replacements that affect the substrate function of the precursor in all instances also drastically reduce the stability of the protein. It appears, therefore, that the C-terminal extension exerts a stabilizing effect on the whole protein during the maturation process (E. Theodoratou and A. Böck, unpublished work).

**Outlook**

The results described in this review give promise that, by a combination of genetic and biochemical approaches, it should become possible to answer the fascinating question of how two transition metals such as iron and nickel are faithfully incorporated into their specific sites in a polypeptide chain, and how the insertion system prevents criss-cross incorporation or the insertion of a non-cognate metal. It is hoped that information will be gained on hitherto unexplored issues, such as how toxic ligands like CO and CN are synthesized, where and how they are bound to the iron, and the way in which ligand stoichiometry is controlled.

A.B. thanks all his students who co-operated in the hydrogenase project, and the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for continuous support.

**References**

Biosynthesis of iron-sulphur clusters is a complex and highly conserved process

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Abstract

Iron–sulphur ([Fe–S]) clusters are simple inorganic prosthetic groups that are contained in a variety of proteins having functions related to electron transfer, gene regulation, environmental sensing and substrate activation. In spite of their simple structures, biological [Fe–S] clusters are not formed spontaneously. Rather, a consortium of highly conserved proteins is required for both the formation of [Fe–S] clusters and their insertion into various protein partners. Among the [Fe–S] cluster biosynthetic proteins are included a pyridoxal phosphate-dependent enzyme (NifS) that is involved in the activation of sulphur from L-cysteine, and a molecular scaffold protein (NifU) upon which [Fe–S] cluster precursors are formed. The formation or transfer of [Fe–S] clusters appears to require an electron-transfer step. Another complexity is that molecular chaperones homologous to DnaJ and DnaK are involved in some aspect of the maturation of [Fe–S]-cluster-containing proteins. It appears that the basic biochemical features of [Fe–S] cluster formation are strongly conserved in Nature, since organisms from all three life Kingdoms contain the same consortium of homologous proteins required for [Fe–S] cluster formation that were discovered in the eubacteria.

Introduction

Nitrogenase is the complex metalloenzyme that catalyses the nucleotide-dependent reduction of atmospheric dinitrogen [1]. The primary translation products of the genes encoding the two catalytic components of nitrogenase, referred to as the Fe protein and the MoFe protein, are not initially active. Rather, their activation requires a consortium of nif-specific genes whose products are required for the formation and insertion of the associated nitrogenase metalcentres [2]. The phenotype arising from inactivation of either nifS or nifU is distinguished from the phenotypes of all of the other nif gene products involved in the formation and maturation of [Fe–S] cluster structures.