

FEMS Microbiology Reviews 27 (2003) 239-261



www.fems-microbiology.org

## Nickel uptake and utilization by microorganisms

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Received 24 September 2002; received in revised form 3 January 2003; accepted 6 January 2003

First published online 26 April 2003

#### Abstract

Nickel is an essential nutrient for selected microorganisms where it participates in a variety of cellular processes. Many microbes are capable of sensing cellular nickel ion concentrations and taking up this nutrient via nickel-specific permeases or ATP-binding cassette-type transport systems. The metal ion is specifically incorporated into nickel-dependent enzymes, often via complex assembly processes requiring accessory proteins and additional non-protein components, in some cases accompanied by nucleotide triphosphate hydrolysis. To date, nine nickel-containing enzymes are known: urease, NiFe-hydrogenase, carbon monoxide dehydrogenase, acetyl-CoA decarbonylase/synthase, methyl coenzyme M reductase, certain superoxide dismutases, some glyoxylases, aci-reductone dioxygenase, and methylenediurease. Seven of these enzymes have been structurally characterized, revealing distinct metallocenter environments in each case.

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Keywords: Nickel sensing; Nickel transport; Nickel enzyme; Metallocenter assembly

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#### 1. Introduction

In 1965, Bartha and Ordal first demonstrated a bacterial growth requirement for nickel while characterizing two strains of hydrogen-oxidizing bacteria [1]. Our knowledge of how these and other microorganisms utilize this trace nutrient has grown tremendously over the ensuing nearly 40 years (reviewed in [2–6]). For example, nine Ni-dependent enzymes are now recognized, with seven being structurally characterized by crystallographic or nuclear magnetic resonance (NMR) approaches. In several cases, activation of these enzymes has been shown to require complex cellular processes utilizing a host of accessory proteins to specifically insert Ni (and other components) into the apoproteins [4,7]. Ni-specific sensing and transport systems also have been characterized in numerous microorganisms [8]. In this contribution, we attempt to summarize recent advances in Ni-sensing, regulation, uptake, and utilization by microorganisms. We focus on cases where nickel has a positive role in the cell, but we also mention a representative example of a Ni-dependent regulatory system associated with Ni-resistance. Additional features of Ni-sensing and Ni-transport related to Niresistance are discussed in greater detail elsewhere in this special issue.

#### 2. Ni-sensing and Ni-dependent regulation

#### 2.1. NikR regulation

Probably the best-characterized Ni sensor/regulator is NikR, a Ni-binding protein that controls the nik operon involved in high-affinity Ni-transport by Escherichia coli. The operon consists of six genes [9] with the first five, nikABCDE, encoding components of a typical ATP-dependent transport system, described in more detail in Section 3.1. The last gene in the operon, nikR, is located 5 bp downstream of the end of nikE, transcribed in the same direction as nikABCDE, and shown to encode a DNAbinding protein that represses transcription of nikABCDE when Ni is present [10]. Two promoters regulate expression of *nikR*. The first is activated by the global *fnr* pathway that controls expression of genes involved in switching from aerobic to anaerobic respiration [11]. The Fnrcontrolled regulation of nikABCDE-nikR occurs at an FNR box located upstream of nikA at a putative NikRbinding site [9]. The second promoter regulating nikR expression occurs 51 bp upstream of the nikR transcription start site and results in low-level constitutive expression. There is also evidence that nikR expression is partially autoregulated [10].

Recent studies by Chivers and Sauer [12–14] and Carrington et al. [15] have provided insight into the structure of the NikR protein. Using profile-based sequence database searches, NikR was shown to be a member of the

ribbon-helix-helix family of transcription factors such as Arc, CopG, MetJ, and Mnt [16-20]. While the N-terminal domain consists of the  $\beta$ - $\alpha$ - $\alpha$  fold characteristic of the DNA-binding domains of this family of regulators, the C-terminal domain of NikR contains a unique His-X<sub>13</sub>-His-X<sub>10</sub>-His-X-His-X<sub>5</sub>-Cys motif of a putative Ni-binding site [12]. The N-terminal DNA-binding domain was cleaved from recombinant His-tagged full-length protein by proteolysis, purified, and biochemically characterized [13]. Circular dichroism spectra revealed this protein fragment is 56%  $\alpha$ -helix, close to the 55% predicted from computational secondary structure predictions. Equilibrium sedimentation experiments showed that the purified domain is a dimer – exactly as predicted for a  $\beta$ - $\alpha$ - $\alpha$ folded fragment. The NikR operator site was defined by DNase footprinting of the nikABCDE upstream region and showed that many of the bases between -32 and +2 are protected. This result demonstrated conclusively that the N-terminal domain of NikR is responsible for binding DNA. Further characterization of the operator site by mutagenesis approaches revealed a two dyad-symmetric 5'-GTATGA-3' recognition sequence. Additional studies were carried out on the purified native full-length NikR. Electrophoretic mobility shift assays confirmed that NikR binds to the upstream region of the nik operon only in the presence of Ni [13]. One of the most interesting conclusions of these studies is that NikR contains two distinct Ni-binding sites, a high-affinity site located in the C-terminal domain that binds stoichiometric amounts of Ni and results in absorbance and circular dichroism spectral perturbations with an apparent  $K_d$  in the pM range, and a low-affinity site that is responsible for DNA binding with a  $K_d$  in the 20–50  $\mu$ M range. The function of the high-affinity Ni-binding site was addressed in studies on the purified recombinant C-terminal domain as well as full-length NikR [14]. Circular dichroism spectroscopy and velocity sedimentation experiments on the C-terminal domain demonstrated that Ni binding induces secondary structural changes, increases the compactness, and increases stability to urea denaturation. Like its full-length counterpart, this domain forms tetramers even in the absence of Ni. The effect of Ni concentration on DNA-binding affinity was examined by using DNase I footprinting and gel mobility shift assays. Binding of Ni at concentrations that allow full occupancy of only the high-affinity sites is sufficient for operator binding, but the affinity for the operator is increased 1000-fold and the operator footprints are larger when both Ni sites are occupied. These results, combined with estimates of intracellular Ni and NikR concentrations, lead to the conclusion that NikR is able to sense and regulate *nik* operon expression over a range of intracellular Ni concentrations from as low as one to as high as 10000 molecules per cell. The authors speculate that occupancy of the high-affinity Ni-binding sites might enhance nik operon expression while occupancy of the low-affinity sites leads to repression.

Although there is no crystal structure of NikR, the nature of the high-affinity Ni site has been characterized by X-ray absorption spectroscopy and mutagenesis experiments. In the absence of DNA, NikR-bound Ni possesses square planar geometry and is likely bound to His87, His89, Cys95, and Glu97 [15]. Upon binding of NikR to operator DNA, the Ni becomes six-coordinate with only N or O ligands (including two histidines) indicating the loss of the Cys ligand. This DNA-dependent structural rearrangement is supported by thiol group titration experiments that reveal one available Cys per molecule of free NikA versus two available Cys per DNA-bound NikR monomer. The exact mechanism by which the N-terminal DNA-binding domain communicates with the C-terminal high-affinity Ni-binding site remains to be elucidated.

Sequence homologues to E. coli NikR are found in a wide variety of other bacteria and archaea [12], and several of these proteins and associated genes have been examined experimentally. For example, the gastric pathogen Helicobacter pylori uses a NikR homologue to modulate expression of its urease genes [21]. Urease is a nickel-dependent enzyme (see Section 4.1) that serves as an essential virulence factor for the microorganism by protecting it from its acidic environment [22]. Expression of H. pylori urease is not regulated by nitrogen availability, urea, growth phase, or pH, as observed in many other bacteria (reviewed in [23]). Rather, Ni regulates urease expression at the transcriptional level in this microorganism [24]. Significantly, a mutant of the nikR gene was isolated that lacks this Ni-dependent regulation [21]. Furthermore, a palindromic operator sequence upstream of the urease promoter is required for Ni-dependent regulation [21,24]. Using a *ureA*::lacZ transcriptional fusion, the response was found to be specific to Ni; however, the ferric ion uptake gene product (Fur) modulates the Ni-dependent response. Regulation of urease by a NikR homolog also has been demonstrated in the human pathogen Brucella suis [25]. In that case, however, the nikR regulatory gene is located upstream of the nikABCDE genes and transcribed in the opposite orientation.

#### 2.2. Other modes of gene regulation by Ni

Although studies with the *nikR* system are the most advanced, significant progress also has been made in understanding other types of Ni-dependent regulation. Here, we briefly review facets of Ni-sensing and regulation associated with certain hydrogenases, *Streptomyces* super-oxide dismutase (SOD), and a representative Ni-resistance system.

Ni-specific regulation is observed for certain microbial NiFe-hydrogenases (see a description of this enzyme in Section 4.2). In pioneering studies involving *Bradyrhi-zobium japonicum*, Ni was shown to stimulate transcription of the hydrogenase genes [26,27]. Follow-up experiments of this plant symbiont identified two proteins, HupU and

HupV, that initially were suggested to form a possible Nisensing regulatory complex necessary for transcription of the hup (hydrogenase uptake) operon [28]. More recent investigations suggest that HupUV might not directly sense Ni, but rather that HypB, a nickel-binding GTPase, incorporates Ni into HupUV to form the active complex [29] that subsequently relays a signal to separate DNAbinding regulatory proteins. The HypB protein contains a histidine-rich N-terminal region that is partially required for Ni binding and stimulation of the hup operon. A B. japonicum strain containing a deletion of the N-terminal 38 residues (23 of which are histidines) exhibits reduced stimulation of hup in 10-50 nM Ni, but wild-type levels of activity are restored by increasing Ni levels to 50 µM. Gel mobility shift assays were used to show that HypB is absent in aerobically grown cells but is expressed in cells grown using microaerobic conditions. Further experiments indicate that the histidine-rich N-terminal region sequesters or stores Ni for subsequent activation of hydrogenase [30].

Insight into the mechanism of downstream signaling for the H<sub>2</sub>-sensing regulatory hydrogenases is available from studies carried out in several microorganisms including Ralstonia eutropha (formerly Alcaligenes eutrophus), Rhodobacter capsulatus, and B. japonicum. For example, R. eutropha HoxBC (the alternative designation for the regulatory hydrogenases in this microbe) interacts with HoxJ, a histidine protein kinase of the HoxJ/HoxA twocomponent regulatory system [31]. Interestingly, activation of this Ni-dependent regulatory hydrogenase [32] is nearly independent of HypB [33]. Analogous two-component systems (e.g., sensor histidine kinases termed HupT) appear to operate in Rhodobacter capsulatus [34] and B. japonicum [35]. The mechanism by which the regulatory hydrogenases modulate activity of the two-component regulatory proteins is unknown. Also unclear is the mechanism by which Ni regulates hydrogenase activity in Rhizobium leguminosarum [36] and two Nostoc strains [37].

In several species of Streptomyces Ni is involved in regulation of SOD genes, functioning as both a positive and negative regulator. Streptomyces coelicolor and Streptomyces griseus have two types of SODs; one contains a Ni cofactor (Ni-SOD, discussed in Section 4.6) while the other contains an iron cofactor (Fe-SOD) [38-41]. The first evidence of Ni-dependent regulation of expression of these enzymes was obtained by Kim et al. [40] who measured Ni-SOD and Fe-SOD activities in conjunction with immunoblotting to show that Ni stimulates the amount of Ni-SOD and decreases the amount of Fe-SOD in S. coelicolor cells. In a subsequent study, Ni-dependent stimulation of transcription of the Ni-SOD gene was documented both by measurement of mRNA levels and by S1 mapping [41]. An unexpected finding was that full-length recombinant Ni-SOD is inactive when expressed in E. coli grown in the presence of Ni, but deletion of the N-terminal 14 residues (yielding the same N-terminus found in the posttranslationally processed enzyme purified from the native organism) resulted in active enzyme. Both the precursor and processed species are observed in recombinant Streptomyces lividans when Ni is not provided, but only the mature form is observed in the presence of Ni. Thus, Ni serves a triple role in Ni-SOD biosynthesis: stimulation of transcription, modulation of post-translational processing, and activation of the enzyme. Genes involved as regulatory element(s) or in post-translational processing have not yet been characterized. In contrast to Ni-dependent stimulation of Ni-SOD transcription, there is also Ni-dependent repression of Fe-SOD in S. coelicolor [42] and S. griseus [43]. In the case of S. griseus, an operator sequence was identified upstream of the transcription start site and gel mobility shift assays using cell extracts showed Ni-dependent binding of an as yet unidentified DNA-binding repressor protein. Similar approaches were used for S. coelicolor where it was also found that Ni does not regulate the amounts of the putative repressor, but only affects its DNA-binding affinity.

Several microorganisms possess heavy metal resistance mechanisms that include regulatory components capable of sensing high concentrations of the specific metal ions. As a representative example, the cyanobacterium Synechocystis sp. PCC6803 possesses a gene cluster involved in resistance to Ni, Co, and Zn [44]. This cluster contains nine open reading frames organized in five transcriptional units that are responsive to extracellular levels of these three metal ions. Four of the genes, nrsBACD, form a single transcriptional unit that is induced by the presence of Ni or Co ions and encode a putative Ni tolerance system designated the Ni response operon (nrs). Subsequent work revealed two adjacent genes, nrsRS, contained in a single transcriptional unit oriented in the opposite direction from *nrsBACD* and forming a two-component signal transduction system [45]. The NrsS protein shares some sequence homology with the alpha subunit of methyl CoM reductase (see Section 4.5) and most likely encodes a periplasmic Ni sensor. The NrsR component is proposed to be a DNA-binding regulatory member of the OmpR/ PhoB family. In the proposed model, the presence of Ni in the medium stimulates the kinase activity of NrsS, which phosphorylates NrsR. Phosphorylated NrsR then binds upstream of the *nrsBACD* operon, activating transcription. This model, however, is yet to be proven experimentally. A homologous two-component system also was proposed to be involved in regulation of Ni-resistance in Legionella pneumophila [46]. Another example of Ni-dependent regulation of expression of Ni-resistance genes is found in the bacterium Achromobacter xylosoxidans 31A [47]. In this organism, *nreB* encodes a Ni efflux transporter and the expression of *nreB* was demonstrated to be Ni dependent. The details of the regulatory elements involved in this system are yet to be delineated. Other contributions to this special issue provide added details regarding Ni sensors associated with resistance mechanisms.

#### 3. Ni uptake

#### 3.1. ATP-binding cassette (ABC)-type transporter systems

Ni is taken up into prokaryotic cells by two types of high-affinity transport systems (reviewed by Eitinger and Mandrand-Berthelot [8]). The first method involves the ABC-type transporters and is described here. The second mechanism makes use of permeases and is described in Section 3.2. The best-characterized ABC-type transporter for Ni is that found in E. coli. This multicomponent system consists of five proteins, NikA-E, that carry out the ATP-dependent transport of Ni [9]. NikA is a soluble, periplasmic, Ni-binding protein; NikB and NikC form a transmembrane pore for passage of Ni; and NikD and NikE hydrolyze ATP and couple this energy to Ni-transport. NikA binds Ni with a  $K_d < 0.1 \mu$ M, but also binds divalent Co, Cu, and Fe with at least 10-fold lower affinity [48]. X-ray absorption fine structure analysis gives best fits to the NikA spectrum by assuming bound Ni is coordinated by 5-7 oxygen or nitrogen ligands at an average bond length of 2.06 Å and one sulfur ligand with a distance of 2.56 A [49]. Although the NikA protein has been purified and crystallized [50], no structure has been reported. The high affinity and specificity of E. coli NikA for Ni ions has been utilized in a practical way to create a fluorescence-based environmental Ni sensor [51].

Selected other microbes are known to contain analogous Ni-specific ABC-type transporters. For example, H. pylori mutants lacking the NixA high-affinity Ni permease (see Section 3.2) retain significant urease activity due to the existence of such a system [52]. In this microorganism, a second Ni-specific transport system was postulated to exist and a four-gene operon designated abcABCD was eventually identified, cloned, and sequenced. Of the four genes, only *abcD* exhibits sequence homology to an *E. coli nik* gene (nikD) while the other three genes have no known homologs [52]. This result demonstrates that other Ni-specific ABC-type transporters may have diverged considerably when compared to the E. coli nik system. Another example is found in the bacterium B. suis [25]. As mentioned in Section 2.1, B. suis urease expression is regulated by a NikR homolog, and this microbe also possesses a nikABCDE operon. The nikA gene of this cluster is not essential for survival of the organism since mutant strains maintain intracellular growth rates comparable to wildtype cells [25]. Furthermore, a potential Ni/Co ABC-type transporter is found in Actinobacillus pleuropneumoniae where a five gene operon, *cbiKLMQO*, displays sequence homology to components of other known ATP-dependent transporters and is needed for full urease activity [53]. Finally, Yersinia pseudotuberculosis, Yersinia pestis, and Yersinia enterocolitica each possess both vntABCDE ABC-type transporters and ureH-type permeases (see below), either of which is sufficient to allow Ni-transport when expressed in E. coli [54].

#### 3.2. Ni-specific permeases

Pioneering work on microbial Ni-transport in R. eutropha was carried out during studies focused on the genetics of hydrogen metabolism. A single gene is responsible for Ni-transport (*hoxN*) and mutants display reduced activity levels of two Ni enzymes, hydrogenase and urease [55–57]. HoxN is an integral membrane protein with a Ni-transport constant (K<sub>t</sub>) of approximately 20 nM [57,58]. Significantly, the transporter concentrates Ni only 10-15-fold and thus functions as a high-affinity, low-capacity system. A topological model, created by analysis of alkaline phosphatase and  $\beta$ -galactosidase fusions, suggests the protein contains eight transmembrane segments [58,59]. More recent studies have shown that HoxN is specific to Ni and unable to transport Co, a finding that contrasts with the NhIF transporter (see below). Site-directed mutagenesis approaches were used to show that His62, Asp67, and His68 of highly conserved transmembrane domain II are critical to permease activity [59]. V64F and N31I variants of HoxN also were created; the former protein exhibits increased velocity for Ni while gaining the ability to transport Co and the latter mutant shows decreased transport of Ni. The authors concluded that the spatial interactions between transmembrane domains I and II control metal ion selectivity [60].

A HoxN homologue known as NixA is found in H. py*lori*, which uses Ni to activate the virulence factor urease (see Section 2.1). In order to isolate gene(s) from this pathogen involved in Ni-transport, E. coli was co-transformed with the recombinant H. pylori urease operon on one plasmid and a H. pylori genomic library on a second compatible plasmid. A library clone containing nixA restored urease activity and was identified as encoding a Ni permease [61]. NixA is an integral membrane protein with a K<sub>t</sub> of 11 nM when expressed in E. coli. Insertional inactivation of nixA in H. pylori resulted in 42% reduced urease activity, with the remaining urease activity arising from the ABC-type Ni-transport system [62] described above. The topology of NixA was examined by using alkaline phosphatase and β-galactosidase fusions and revealed that, like HoxN, there are eight transmembrane segments [63]. Furthermore, the fusion constructs also indicate that regions of transmembrane domains II and III are essential for transport. Chemical crosslinking experiments on isolated membranes show that NixA is most likely a monomeric protein. Site-directed mutagenesis of conserved residues demonstrated that Phe75 and His79 in transmembrane domain III, Asn127 in transmembrane domain IV, and Thr195 and Ser197 in domain V are all essential for Ni-transport [64]. Furthermore, five other residues not believed to interact directly with Ni were shown to greatly decrease Ni-transport [65].

Additional homologues to HoxN encoding single peptide Ni permeases have been investigated in other bacteria and a fission yeast. One of the best-characterized HoxN homologues is found in Rhodococcus rhodochrous J1 where NhIF was initially identified as a Co-specific transporter [66], but later shown to transport both Co and Ni with a slight preference for Co [67]. The transport process is energy-dependent since energy uncouplers inhibit transport, but the energy source that drives the transport process remains unknown. Recent work on NhIF has focused on sequence comparisons with HoxN and examination of conserved regions in transmembrane domains I and II. Substitution by site-directed mutagenesis of conserved His residues in domain II of NhIF decreases both Ni and Co transport [60]. Similar studies investigating the correlation of a hydrophobic Phe residue in domain II and a His residue in domain I show that replacement of the His with an Ile eliminates all transport activity, while replacement of the Phe with Val greatly reduces both Ni and Co transport. These studies, in conjunction with domain-swapping experiments between HoxN and NhlF, provide further evidence that intricate spatial interactions between transmembrane domains I and II play a large part in metal ion selectivity of these permeases [60]. Another notable HoxN homolog was identified in the fission yeast Schizosaccharomyces pombe genome sequence [68]. Interruption of this chromosomal gene, designated nic1, results in a urease negative phenotype. Many individual metal ions were tested as inhibitors of Ni uptake in the wild-type yeast, but only Co shows significant reduction. The gene product, Niclp, is proposed to be a transmembrane Ni-transporter. These findings raise the possibility of Ni-transporters in other higher eukaryotes [68].

Other Ni permeases also are known. In particular, HupN of B. japonicum has 56% identity to HoxN and its gene complements a Ni metabolism-defective mutant strain [69]. Similarly, UreH is encoded by the urease gene cluster of a thermophilic Bacillus sp. and shares 23% sequence identity with HoxN [70]; however, no further analysis of this gene product has been carried out. Homologous proteins are produced in Y. pseudotuberculosis, Y. pestis, and Y. enterocolitica, and in one case the recombinant protein was shown to allow Ni uptake by E. coli [54]. Finally, evidence for Ni permeases has been uncovered in other microbes by various types of transport studies. As one recent example, studies using Rhodospirillum rubrum have characterized Ni-transport by using nickel-containing carbon monoxide dehydrogenase (CODH, a Ni enzyme described in Section 4.3) activity as a functional assay [71]. Ni-transport into these cells is partially inhibited by Co<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, but not Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ca<sup>2+</sup>, or Zn<sup>2+</sup>. More significantly, Ni-transport is also partially inhibited by cold and by protonophores, whereas ATP synthase inhibitors and incubation of cells in the dark stimulates transport two-fold [71]. Further studies will be needed to identify the gene associated with this activity and to define the protein(s) associated with transport.

#### 4. Ni enzymes and metallocenter assembly systems

#### 4.1. Urease and urease accessory proteins

Urease catalyzes the hydrolysis of urea to yield ammonia and carbamate, which spontaneously decomposes to yield carbonic acid and another molecule of ammonia (Eqs. 1 and 2). In many microorganisms the released ammonia serves as a nitrogen source, allowing growth on urea precursors such as arginine and purines [72]. In other cases, the enzyme is a virulence factor that participates in gastroduodenal infection by *H. pylori*, urinary stone formation by many bacteria including *Proteus mirabilis*, ammonia encephalopathy, and other human and animal disease states (reviewed in [23,72]).

$$\begin{array}{c} O \\ H_2N \\ H_2N \\ H_2N \\ H_2N \\ H_2N \\ OH \end{array} + H_2O \\ H_2O \\ H_2O \\ H_2CO_3 \\ H_3CO_3 \\ H_3 \\ H$$

Urease has the distinction of being the first enzyme shown to contain Ni [73]. Although the protein used in this initial discovery was isolated from soybean seeds, all bacterial, algal, fungal, and plant ureases subsequently examined contain this metal ion (see [74] for a recent review). The structure of the dinuclear Ni active site (Fig. 1A) has been resolved by crystallographic methods using enzymes from Klebsiella aerogenes [75,76], Bacillus pasteurii [77], and H. pylori [78]. Significantly, a modified amino acid side chain - a carbamylated lysine residue bridges the two metal atoms. Site-directed mutagenesis studies involving K. aerogenes urease revealed that the carbamate is not essential for catalysis and can be replaced by a non-covalently bound organic acid molecule in mutants lacking this lysine [79]. Also bridging the two Ni ions is a water molecule that likely plays a critical catalytic role [74]. Three of these dinuclear active sites are present in the K. aerogenes and B. pasteurii proteins (comprised of (Ure-ABC)<sub>3</sub> structures, with UreA  $\sim 11$  kDa, UreB  $\sim 12-14$ kDa, and UreC ~60–61 kDa). In contrast, the H. pylori enzyme has a  $\sim 26.5$  kDa UreA peptide (corresponding to a fusion of the two typical small units) and a  $\sim 61.7$  kDa UreB peptide, with four (UreAB)<sub>3</sub> trimers of dimers forming a supermolecular assembly [78,211]. Eukaryotic ureases are homopolymeric with their single  $\sim$  90-kDa subunits, representing a fusion of each of the UreA, UreB, and UreC peptides, assembled into trimeric or hexameric structures. Despite these differences in quaternary structure, all urease sequences are highly conserved and the metallocenters are thought to be identical. The metal ions appear to be deeply buried in the enzyme and inaccessible to chelators, raising the question of how the metals and the carbamate become incorporated into the protein.

Synthesis of the urease metallocenter is a complex process requiring Ni, carbon dioxide (used for carbamylation), several accessory proteins, and GTP hydrolysis (reviewed in [80]). Our current understanding of the key events in cellular urease activation is illustrated in Fig. 2. The process initiates with ribosomal synthesis of urease apoprotein and cellular chaperonins participating in protein folding, as shown by studies involving the H. pylori GroES homologue [81]. Crystallographic studies reveal the overall structure of K. aerogenes urease apoprotein is identical to that of native enzyme, but lacks Ni and carbamylation [82]. The apoprotein from this microorganism forms a series of complexes with three essential urease accessory proteins: UreD, UreF, and UreG [83-86]. Of particular interest, in vitro activation studies reveal increasing extents of urease activation when using apoprotein alone [87] versus UreD-urease apoprotein [88], UreD-UreF-urease apoprotein [84], and UreD-UreF-UreG-urease apoprotein [89]. Significantly, the latter species exhibits GTP-dependent urease activation, associated with a nucleotidebinding site located on the UreG component, when using physiologically significant concentrations of Ni and bicarbonate ions [89]. Evidence has been presented for the presence of similar accessory protein:urease complexes in H. pylori and P. mirabilis. For example, yeast two-hybrid analyses of the H. pylori proteins identifies interactions between UreD (alternatively named UreH in this organism; not to be confused with UreH Ni permeases in several microbes) and both UreA and UreF [90,212]. Similarly, in vitro immunoprecipitation studies and yeast twohybrid analysis reveal UreD:UreC and UreD:UreF interactions in the P. mirabilis enzyme [91]. As shown in Fig. 2, we propose that the UreDFG-urease apoprotein complex plays a critical role in urease activation.

Ni delivery to the UreDFG-urease apoprotein complex is proposed to occur via the Ni metallochaperone, UreE. This urease accessory protein binds Ni, although the number of metals bound varies depending on the source. For example, the K. aerogenes protein binds  $\sim 6$  Ni per dimer [92] whereas B. pasteurii UreE binds a single Ni per dimer [93]. These differences arise in part from the presence of a histidine-rich region at the carboxyl-terminus of the former protein. By using site-directed mutagenesis methods to create a truncated K. aerogenes UreE protein, the Hisrich region was shown to be non-essential for metallochaperone function [94]. Three-dimensional structures of the truncated K. aerogenes protein [95] and full-length B. pasteurii UreE [96] reveal nearly identical protein folds, with a conserved Ni-binding site located at the interface of the dimer. Surprisingly, the Ni-binding domain of UreE closely resembles the structure of the Cu-binding Atx1 metallochaperone [97]. In addition, both UreE proteins contain a second module that structurally resembles a domain of an Hsp40-type chaperone protein [98]. A reasonable hypothesis, still to be tested, is that the chaperone-like module participates in UreE docking to its partner protein



Fig. 1. Active sites of structurally defined Ni-containing enzymes. A: Dinuclear Ni active site of K. aerogenes urease (PDB code 1FWJ) [76]. The carbamylated lysine is shown as K217\* and metal-bound waters as red spheres. Analogous sites have been crystallographically defined for ureases from B. pasteurii (PDB code 2UBP) [77] and H. pylori (PDB code 1E9Z) [78]. B: Dinuclear Ni-Fe active site of the reduced, active hydrogenase from D. norvegium (formerly D. baculatum) (PDB code 1CC1) [111]. The diatomic molecules bound to Fe are CO and two cyanide molecules. In some states of the enzyme an additional bridge connects the Ni and Fe sites. Structures also are known for hydrogenases from D. gigas (PDB code 2FRV) [106,107], D. vulgaris Miyazaki (1H2A) [108,109], D. fructosovorans (1FRF) [110], and D. desulfuricans (1E3D) [112]. C: C. hydrogenoformans CO dehydrogenase active site (PDB code 1JJY) [151]. The corresponding site in the R. rubrum enzyme (PDB code 1JQK) is slightly altered, with one less sulfide, an extra bonding interaction between the Ni-bound cysteine and the unique Fe, and a possible CO ligand [152]. D: One proposal for the ACDS active site of M. thermoacetica (formerly C. thermoaceticum) (PDB code 1MJG) [165]. A 67-Å-long molecular tunnel connects this active site to a Ni-containing CODH active site elsewhere in the protein. AYT refers to a possible acetyl ligand on the Cu. An alternative structure of this same enzyme (PDB code 10AO) has Cu replaced by a second Ni [215], consistent with composition and spectroscopic studies of one component of the recombinant M. thermophila enzyme [169]. E: Ni-containing tetrapyrrole of methyl coenzyme M reductase [according to structures of the enzymes from M. marburgensis (formerly M. thermoautotrophicum strain Marburg), M. barkeri, and M. kandleri; PDB codes 1MR0, 1HBN, and 1E6V, respectively] [176-178]. A glutamine residue coordinates the metal from below the plane of the ring, and substrate interacts with the metal from above the plane. F: Active site of E. coli glyoxylase with two bound water molecules (PDB 1F9Z) [197], with the prime symbol designating the second chain of the dimeric protein. G: Active site of the Ni-containing form of K. pneumoniae aci-reductone dioxygenase (PDB 1M4O) derived from homology modeling to the cupin family after obtaining most of the protein solution structure [203]. One of the histidyl ligands (yet unidentified) coordinates the metal via its No1 atom rather than the Nɛ2 atoms shown. All Ni atoms are shown in green, Fe atoms in pink, Cu in cyan, S in yellow, and Se in light blue.



### Urease Holoprotein

Fig. 2. Model of urease activation. Synthesis of active urease requires the formation of a complex between the  $(UreABC)_3$  urease apoprotein and UreDFG. This complex accepts Ni from the UreE metallochaperone (e.g., PDB codes 1CFZ and 1EBO for the *K. aerogenes* and *B. pasteurii* proteins, respectively) and inserts it into the carbamylated apoenzyme (thus the requirement for CO<sub>2</sub>) in a GTP-dependent reaction. Dissociation of the accessory proteins results in active enzyme (e.g., three copies of the UreABC trimer for the *K. aerogenes* enzyme shown, PDB code 1FWJ).

while the metal-binding domain functions in Ni transfer to the urease apoprotein. Consistent with an interaction of UreE and the UreDFG–urease apoprotein complex, yeast two-hybrid experiments indicate an association between UreE and UreG in *H. pylori* [90,212]. Furthermore, in vitro activation studies demonstrate that urease within the Ure-DFG–urease apoprotein complex is fully activated only by the inclusion of UreE [99]. Of particular interest, studies reveal that UreE functions even in the presence of chelators; hence, UreE is not simply a reversible carrier of Ni in this process [99]. The CO<sub>2</sub>, Ni–UreE, and GTP-dependent activation process results in production of urease holoprotein and dissociation of the accessory proteins (Fig. 2).

In contrast to the persuasive evidence supporting a Nidelivery function for UreE, the precise roles of UreD, UreF, and UreG in urease activation remain unclear. One reasonable possibility is that the proteins act together as a GTP-dependent protein chaperone that alters the conformation of urease apoprotein to increase accessibility to the buried active site. Alternatively, roles in CO<sub>2</sub> activation, establishing proper docking of UreE to urease, selecting for Ni versus other transition metals, or other functions remain to be experimentally tested. These four accessory proteins generally are encoded adjacent to the urease structural genes in bacteria, although their precise order can vary (reviewed in [23]), the accessory genes may be fused (e.g., ureE and ureF are joined in Bordetella bron*chiseptica* [100]), or these genes may be interrupted by other open reading frames (e.g., as found in R. leguminosarum [101]). Notably, the fission yeast S. pombe also synthesizes UreD, UreF, and UreG homologues that are required for urease activation [102], however the genes encoding these proteins are distant from that encoding the single-subunit enzyme. Higher eukaryotes exhibiting urease activity also contain homologues of these genes, with a soybean gene complementing an S. pombe ureF mutant [102] and a potato gene complementing (poorly) a K. aerogenes ureG deletion [103].



Fig. 3. Model for activation of Ni-containing hydrogenases and  $H_2$  sensors. The large subunit (LS) of NiFe-hydrogenases is initially synthesized as a precursor containing a carboxyl-terminal extension (pre-LS). This protein becomes bound to a hydrogenase-specific chaperone protein (e.g., HypC shown here) that is presumed to alter the pre-LS conformation so as to allow access to the active site. Prior to forming this species, the chaperone protein is in a complex with HypD that acquires Fe (by an unknown mechanism) along with its one CO and two CN ligands (the three diatomic molecules are shown by the three lines). HypE and HypF (or their homologues) are needed for forming the cyanide (and perhaps CO) ligands from CP in reactions that require ATP and reductant, perhaps shuttled through the Fe–S cluster of HypD. HypC then delivers the Fe and its ligands to pre-LS, while releasing HypD. Addition of Ni occurs in a GTP-dependent step that requires HypB and HypA (or their analogues). Dissociation of the chaperone protein allows action of a specific protease (e.g., the structurally characterized *E. coli* HybD, PDB code 1CFZ) that removes the carboxyl-terminus. The mature length LS binds to the small subunit (SS), which also must undergo processing events including the incorporation of several Fe–S clusters, to form the functional enzyme (e.g., the *D. fructosovorans* hydrogenase shown, PDB code 1FRV). The H<sub>2</sub> sensor or regulatory hydrogenase (RH) is synthesized without the carboxyl-terminal extension; thus, a hydrogenase-specific chaperone and a protease are not involved. Rather, activation involves HypDEF to incorporate Fe with its ligands, HypAB to insert Ni, and binding to the SS.

In addition to possessing homologues of the *K. aero-genes*-type urease accessory proteins, some bacteria utilize other gene products for urease activation. For example, urease gene clusters of a thermophilic *Bacillus* sp. [70] and several *Yersinia* species [54] encode Ni permeases, as described in Section 3.2. Furthermore, urease activation in *H. pylori* is further complicated by the involvement of two hydrogenase accessory proteins. Interruption of either *hypA* or *hypB* leads to the cessation of hydrogenase activity [104]. Supplementing the medium with excess Ni ions restores these activities. The effects were shown to arise from deficiencies in incorporating Ni into urease rather than reduc-

tions in urease transcription. Purified *H. pylori* HypA binds 2 Ni homodimer and the corresponding HypB homodimer is a GTPase [213]; however, the roles of HypA and HypB in activation of *H. pylori* urease, and the involvement of these proteins in urease activation within other microorganisms, remain to be defined.

#### 4.2. NiFe-hydrogenase and hydrogenase accessory proteins

Hydrogenases play a central role in microbial energy metabolism by catalyzing the reversible oxidation of hydrogen gas (Eq. 3). Hydrogen production is a mechanism used to vent excess reducing potential in some anaerobic microorganisms, while hydrogen consumption can be coupled to reduction of oxygen, sulfate, carbon dioxide, or other electron acceptors and used to generate a proton motive force in diverse species. The identity of the associated electron carriers varies widely with the source of the enzyme, ranging from cytochromes to ferredoxins, nicotinamides, or other cofactors. As recently reviewed [105], three phylogenetically distinct classes of hydrogenases are known: NiFe-hydrogenases, Fe-hydrogenases, and metal-free hydrogenases. The former grouping contains the vast majority of these enzymes, with the name denoting the presence of Ni and Fe at the active site. The requirement for Ni in these enzymes explains the observation of Ni-dependent chemolithotrophic growth of two strains of hydrogen-oxidizing bacteria [1] that was mentioned in Section 1.

$$H_2 \rightleftharpoons 2H^+ + 2e^- \tag{3}$$

The structure of the unique dinuclear Ni-Fe active site in NiFe-hydrogenases has been revealed by crystallographic studies of the proteins isolated from Desulfovibrio gigas [106,107], Desulfovibrio vulgaris Miyazaki [108,109], Desulfovibrio fructosovorans [110], Desulfomicrobium norvegium (formerly Desulfomicrobium baculatum) [111], and Desulfovibrio desulfuricans [112]. Four cysteine residues (or three Cys plus a selenocysteine in the D. norvegium enzyme, as illustrated in Fig. 1B) coordinate the Ni, with two of these Cys also binding the Fe atom. In addition, the Fe possesses three non-protein diatomic ligands, identified as one carbon monoxide and two cyanide groups in D. gigas and Allochromatium vinosum (formerly Chromatium vinosum) [107,113] or as SO, CO, and cyanide in D. vulgaris [108,114]. The structure shown represents the reduced, active form of the enzyme, whereas alternative states of these proteins additionally possess an oxo or sulfido group bridging the Ni and Fe atoms [109,111]. Elsewhere in the proteins are multiple Fe–S clusters that form a conduit for electron transfer to the appropriate electron carrier. Perhaps not unexpectedly, an intricate pathway involving numerous helper proteins is required for biosynthesis of these enzymes.

Discussion of the hydrogenase maturation systems is confounded by the variety of gene/protein designations used in different microorganisms and the presence of multiple versions of hydrogenases and auxiliary proteins in some species [105,115,116]. All NiFe–hydrogenases possess a minimum of two distinct peptides, referred to as the small subunit (SS) and the large subunit (LS). For example, *hyaAB*, *hybOC*, and *hycGE* encode three *E. coli* hydrogenases (a fourth appears to be encoded by *hyfIG* [117], but its activity could not be detected [118]), *hoxKG* and *hoxYH* encode two *R. eutropha* enzymes, and alternative designations (e.g., *hupSL*, *hynAB*, *hydAB*, *echCE* named for energy-coupling hydrogenase, and many more) are used in assorted other microorganisms [105,115]. Similarly, hydrogen-sensor proteins possess two components (e.g., those encoded by hupUV in R. capsulatus or hoxBC in R. eutropha) that interact with histidine kinase and response regulator proteins to modulate transcription of the hydrogenase-related genes in selected species (e.g., [28,34,119]). The H<sub>2</sub>-sensor proteins, clearly related in sequence to the hydrogenase subunits, possess a  $Ni-Fe(CO)(CN)_2$  active site according to spectroscopic studies [32] and exhibit hydrogen/deuterium exchange activities [31,120,121]; however, they are very poor catalysts of hydrogen oxidation or production. As described below, the H<sub>2</sub>-sensor proteins require only a subset of the accessory proteins required for maturation of the true hydrogenases. As in the case of the hydrogenase structural proteins, the accessory proteins are known by more than one designation in distinct organisms and there can be multiple copies within the same organism. In the following paragraphs we describe reasonable working hypotheses for maturation of NiFe-hydrogenases and NiFe-hydrogen sensors (illustrated in Fig. 3).

Hydrogenase biosynthesis begins with ribosomal translation of the gene encoding the LS to form a precursor protein (pre-LS) with a  $\sim 15$  residue extension at the carboxyl-terminus. On the basis of both diminished hydrogenase activity in groEL and groES E. coli mutants and specific binding of GroEL to pre-LS [122], proper folding of the pre-LS peptide is likely to be facilitated by the GroE chaperonins. An early maturation step involves formation of a protein complex between a protein chaperone and pre-LS (e.g., HypC:pre-HycE, HybG:pre-HybC, HypC: pre-HyaB, and HybG:pre-HyaB are formed in E. coli [123,124], with HypC shown in the figure), where the chaperone protein likely confers a distinct conformation to pre-LS compared to the non-complexed protein. Significantly, complex formation requires three structural features: the carboxyl-terminal extension of the subunit precursor, one of the hydrogenase cysteines that eventually becomes a Ni ligand, and the cysteine at the amino-terminus of the chaperone protein [124,125]. Truncated protein lacking the carboxyl-terminal extension cannot be activated, as demonstrated for both an E. coli hydrogenase [126] and one of the *R. eutropha* enzymes [127].

The chaperone:pre-LS complex is suggested to contain the active site Fe already possessing its CO and CN ligands. HypDEF (alternatively named HupDEY in some other species) play key roles in this assembly. The Fe donor is not identified, but the metal ion appears to be incorporated into a complex formed by HypC and HypD [128]. Synthesis of the CN (and perhaps also CO) ligands is accomplished by HypF and HypE [214]. Experiments with an *E. coli carAB* mutant, deficient in carbamoylphosphate (CP) synthesis, provide critical insight into this process by demonstrating a requirement for CP in hydrogenase activation [129]. Additional experiments reveal that purified HypF both hydrolyzes CP to release inorganic phosphate and catalyzes CP-dependent pyrophosphate exchange into ATP [130]. More significantly, HypF catalyzes the ATP-dependent transfer of the CP carbamoyl group to the carboxy-terminal cysteine of HypE according to mass spectrometric analyses [214]. The intrinsic ATPase activity of HypE [116] is coupled to a dehydration reaction, resulting in HypE that is cyanated at the C-terminus [214]. The protein-bound cyano group is suggested to provide the CN ligand of the Fe. The CO biosynthetic pathway is less clear, but this ligand may arise from hydrolysis of the CN ligand followed by deamination. Reflecting some of this potential chemistry, HypF contains sequence motifs characteristic of O-carbamoyl transferases [129] and acylphosphatases (this domain of HypF has been crystallized and structurally characterized [131]). Also of potential interest, the HypE sequence resembles that of aminoimidazole ribonucleotide synthetase and thiamine phosphate kinase, enzymes that catalyze somewhat related chemical steps [130]. Transfer of the protein-bound ligands to Fe requires a source of reductant. In this regard, it is interesting that HypD contains an Fe-S cluster [116]. After the CN and CO ligands are bound to Fe, HypC is suggested to deliver this portion of the final metallocenter to pre-LS [128] (Fig. 3).

The next step in hydrogenase biosynthesis is the GTPdependent insertion of Ni into the Fe(CO)(CN)2-containing chaperone:pre-LS complex, an event involving at least two accessory proteins. The nucleotide-binding accessory protein required for this process (HypB or HupB) is related in sequence to one of the urease accessory proteins (UreG) and exhibits low levels of GTPase activity [132-135,213]. In some microorganisms (e.g., R. leguminosarum [135] and *B. japonicum* [134]) this protein possesses a histidine-rich motif and binds multiple Ni atoms (reminiscent of the situation for some UreE proteins involved in urease activation), but other representatives of this protein (e.g., that in E. coli) lack such a motif. Deletion of the His-rich region from the *B. japonicum* protein leads to only modest effects on its ability to insert Ni into hydrogenase while severely affecting the ability of the cell to store Ni [29,30]. The B. japonicum protein lacking the His-rich region still binds one Ni per monomer, consistent with a direct Niinserting role for this protein; however, Ni binding has not been reported for the homologous E. coli protein. In this organism Ni might alternatively bind to the second accessory protein required for the metal insertion step. Indeed, H. pylori HypA is known to bind two Ni per dimer [213]. HypA is alternatively termed HupA is some microorganisms, and a second copy called HybF exists in E. coli (where it is specific to activation of hydrogenase-1 and hydrogenase-2) [136]. Persuasive evidence has been reported for cooperation between HypA and HypB during insertion of Ni into hydrogenase (e.g., [136]), and a heterodimer of the *H. pylori* proteins was detected by chemical crosslinking studies [213]. Moreover, a connection between these components was noted in H. pylori where both proteins are required for generating active urease and hydrogenase [104]. Although many questions remain unanswered about the specific steps of the process, HypA/HypB-dependent Ni insertion into the hydrogenase precursor appears to result in a chaperone:pre-LS complex containing both the Ni and the  $Fe(CO)(CN)_2$  centers (Fig. 3).

The final steps in hydrogenase biosynthesis include chaperone dissociation, proteolytic removal of the carboxyl-terminus, and binding of the processed LS to SS. In studies focused on one hydrogenase in E. coli, dissociation of the chaperone from the Ni-containing pre-LS was shown to precede the proteolytic step [137]. Each of the E. coli pre-LS proteins is processed by a distinct protease (HyaD for pre-HyaB, HybD for pre-HybC, and HycI for pre-HycE), cleaving at a position located a few residues beyond the last Ni ligand in the sequence. The structure of one such protease (HybD) has been elucidated and found to topologically resemble the metzincin protease superfamily [138]. Of particular interest, this protease possesses a metal-binding site that has been suggested to interact with Ni bound to its cognate pre-LS, accounting for a key feature of its substrate recognition motif [139]. Further supporting this notion, Zn prevents proteolysis of the pre-LS and hinders formation of a protease:pre-LS complex by substituting for Ni [140]. Only after proteolysis is the LS capable of binding to the SS [137]. The latter protein must also undergo maturation events, including insertion of Fe-S clusters and, in many cases, processing of its amino-terminal signal sequence.

Maturation of the H<sub>2</sub> sensor proteins (also known as regulatory hydrogenases, RHs) appears to involve a slightly less complicated situation. These proteins lack the carboxyl-terminal extension found in the true hydrogenases; thus, no protease is needed and interaction with a hydrogenase-specific chaperone is hindered. Rather, incorporation of Fe(CO)(CN)<sub>2</sub> and Ni is likely to occur directly into the RH-LS. Maturation of the RH in *R. capsulatus* was shown to require at least HypD [141] and HypF [142]. Similarly, synthesis of the functional *R. eutropha* protein is highly dependent on HypDEF, although partial dependency on HypAB and, surprisingly, HypC was noted when the protein is highly overproduced in the cell [33]. As illustrated in Fig. 3, we presume that binding of the RH-SS is the final step in synthesis of this protein.

In addition to the accessory proteins noted above, two other gene products are proposed to participate in activation of some hydrogenases. HupK, alternatively designated HoxV or HupV, was first noted in *R. leguminosarum* [143] and has since been found in a subset of hydrogenasecontaining microorganisms. The sequence of this protein resembles that of the LS, but lacks two of the four cysteines that function as Ni ligands. This feature served as the basis for the suggestion that HupK acts as a scaffold to support synthesis of a metal site that is subsequently transferred to the LS [143]. Although this suggestion remains an intriguing notion, the absence of a comparable protein in some hydrogenase-containing microorganisms and the retention of 30% of the hydrogenase activity upon mutating the R. eutropha hoxV gene [144] call into question this proposal. A similar situation holds for the case of HypX, also known as HoxX. The R. leguminosarum protein possesses sequences with similarities to tetrahydrofolate-dependent enzymes and to enoyl-CoA hydratase/isomerases [145]. The former observation led to the proposal that HypX is involved in delivery of the CO and cyanide ligands of the Fe, presumably as formimino and formyl groups bound to the one-carbon carrier. As in the case of HupK, homologues to HypX are absent from many hydrogenase-containing species and hoxX mutants of B. japonicum retain nearly half of hydrogenase activity compared to wild-type cells [146]. Potential roles of HupK and HypX proteins are not included in the activation scheme shown in Fig. 3.

# 4.3. Carbon monoxide dehydrogenases (CODHs) and its accessory proteins

CODHs catalyze the reversible oxidation of carbon monoxide to carbon dioxide (Eq. 4). Two distinct types of such enzymes, readily distinguished by their cofactor content and source, have been identified. This review ignores the molybdenum–molybdopterin cytosine dinucleotide–[2Fe–2S]–FAD-containing proteins isolated from a few aerobic microorganisms [147]. Rather, we describe the Ni–Fe–S-containing enzymes that carry out this chemistry in a variety of anaerobic microorganisms [148–150]. This section focuses on enzymes that only possess CODH activity, whereas Section 4.4 details the more complex CODH/acetyl–CoA decarbonylase/synthases (ACDS) that possess dual activities. The presence of CODH activity allows for growth on carbon monoxide as sole carbon and energy source.

$$CO + H_2O \rightleftharpoons CO_2 + 2e^- + 2H^+ \tag{4}$$

Crystal structures are known for homodimeric CODHs from Carboxythermus hydrogenoformans [151] and R. rubrum [152]. Both structures reveal the presence of a [4Fe-4S] center (site D) bridging the subunits and likely serving as the site of electron transfer to an external electron carrier protein. Two [4Fe-4S] centers (sites B and B') are positioned appropriately to function as conduits for electrons to site D from Ni-Fe-S centers located in the opposite subunit (sites C' and C). The precise structure of the C site differs in the two proteins (or may vary depending on how the proteins are isolated), but appears to be comprised of a [1Ni-3Fe-4S] center fused with a mononuclear Fe site. An additional bridging sulfide is found in the 1.6-Å structure of the C. hydrogenoformans enzyme (illustrated in Fig. 1C), whereas the 2.8-A structure of the R. rubrum enzyme shows no bridging sulfide, but has an additional bonding interaction between the Ni-coordinated cysteine and the unique Fe along with a possible CO molecule bound to the Ni. Radiolabeling studies previously had provided biochemical evidence for the presence of a non-substrate CO ligand in the *R. rubrum* protein, but those results indicate binding of CO to Fe rather than Ni [153]. Significantly, prior spectroscopic studies had been interpreted to suggest that a separate Ni site was bridged to a [4Fe-4S] center (see references in [148,149]), whereas both of these structures position the Ni within the cubane and place one Fe at a unique site.

Information regarding the mechanism of Ni insertion into CODH is available only from studies with the R. rubrum enzyme. Inactive CODH, purified from cells grown in the absence of Ni, can be transformed into active enzyme by first reducing a pre-existing Fe-S cluster and then simply adding Ni, which binds with a  $K_d$  of 755  $\mu$ M [154,155]. The non-physiological Ni concentration needed for these in vitro studies is consistent with a requirement for accessory components during CODH activation in the cells. Genetic and physiological studies reveal that cooCTJ, located downstream of the structural gene cooS, encodes at least two proteins that facilitate Ni insertion [156]. One required protein for CODH activation is CooC, a dimeric protein that binds neither Ni nor Fe, but contains an essential nucleotide-binding motif [156,157]. As described in Sections 4.1 and 4.2, the nucleotide-binding proteins UreG and HypB are similarly required for urease and hydrogenase activation. Purified CooC exhibits both ATPase and GTPase activities with similar  $K_{\rm m}$  values for the two substrates, but a 15-fold higher catalytic rate with ATP [157]. The 66-residue CooT has no obvious functional motifs and, significantly, has not been clearly shown to be involved in CODH activation [156]. The second required protein for activation is CooJ, a 115-residue protein that is especially rich in histidine residues at its carboxylterminus (16 His in the last 34 residues). Again this situation is reminiscent of urease and hydrogenase activation, where His-rich accessory proteins include UreE and, from some sources, HypB. Of no surprise, CooJ is a Ni-binding protein (4 Ni/monomer) and binds this metal with a  $K_d$  of 4.3  $\mu$ M [158]. Although the His-rich region of this protein is non-essential to its function [156], no studies report whether the truncated protein still binds Ni. The precise roles of the CODH accessory proteins remain unknown, but a scheme illustrating our current understanding of this system is provided in Fig. 4.

#### 4.4. Acetyl-coenzyme A decarbonylaselsynthase

In addition to the independent CODH enzymes described in Section 4.3, CODH activity also occurs as one of two distinct Ni-dependent activities associated with ACDS that are found in selected methanogenic, sulfatereducing, and acetogenic microorganisms [148–150]. ACDS functions to decompose the acetyl group into separate one-carbon units in some cells (e.g., see [159,160] and references therein) or to catalyze acetate synthesis from



Fig. 4. Model of CODH activation. Proteins that are non-specific to the CODH system are likely required to convert apoprotein into the dimeric protein containing its full complement of Fe–S clusters (the bridging [4Fe–4S], two [4Fe–4S] in each subunit, and the Ni-free precursor to the [1Ni–4Fe–4S] or [1Ni–4Fe–5S] cluster in each subunit). Ni insertion likely involves an ATP-dependent step catalyzed by CooC along with metal delivery by CooJ. Activation is completed upon binding of a non-substrate molecule of CO. The structure shown is that of the enzyme isolated from *R. rubrum* (PDB code 1JQK).

one-carbon precursors in others (e.g., see [148,161,162]), but all of these enzymes are capable of the reversible reactions shown in Eqs. 4 and 5. In acetate-degrading organisms, such as Methanosarcina barkeri or Methanosarcina thermophila, the reaction shown in Eq. 5 proceeds to the right at one Ni-containing active site with acetyl-CoA being split into CoA, a methyl group that becomes bound to a corrinoid-iron-sulfur protein (abbreviated Co(I)-FeSP), and CO. The CO molecule then is oxidized to CO<sub>2</sub> via Eq. 4 at a second Ni-containing active site. The corrinoid-bound methyl group is sequentially transferred to a reduced pterin analogue (tetrahydrosarcinapterin or tetrahydromethanopterin) and coenzyme M, eventually forming methane after being reduced using the electrons available from Eq. 4 (methyl-coenzyme M reductase, required for this step, is described in Section 4.5). In contrast, acetogenic organisms such as Moorella thermoacetica (formerly Clostridium thermoaceticum) use the reactions in reverse. Thus, CO<sub>2</sub> is reduced to CO that is joined with CoA and a corrinoid-bound methyl group, derived from methyl-tetrahydrofolate, to form acetyl-CoA. Of particular interest, biochemical evidence suggests a molecular tunnel connects the CODH active site and the acetyl-CoA-binding site (i.e., the C- and A-clusters, respectively, present in distinct subunits of the M. thermoacetica enzyme) and allows for direct transfer of CO [163,164]. All enzymes in this class presumably utilize a similar channeling approach to couple the two Ni-containing active sites. The crystal structure of the dual-activity M. thermoacetica ACDS demonstrates the existence of such a tunnel that is remarkably 67 Å long [165]. The CODH (C-cluster) active site of this enzyme is found in the  $\beta$ -subunit that structurally resembles the C. hydrogenoformans and R. rubrum enzymes [151,152]. In contrast, the M. thermoacetica  $\alpha$ -unit has a distinct 3-domain structure and contains the unique A-cluster. Selected properties of the unique A-cluster, derived from biochemical, spectroscopic, and crystallographic approaches, are discussed below.

$$CH_{3}C(O) - S - CoA + Co(I) - FeSP \rightleftharpoons$$

$$CH_{3} - Co(III) - FeSP + CO + CoA$$
(5)

The Ni site associated with the A-cluster is structurally and spectroscopically distinct from Ni in the C-cluster of ACDS. For example, addition of 1,10-phenanthroline to the *M. thermoacetica* enzyme results in selective Ni loss from the A-cluster with resulting loss of acetyl-CoA synthase activity, but no effect on CODH activity [166]. Full activity is restored upon subsequent addition of Ni ions. Spectroscopic characterization highlights a unique NiFeC EPR signal of the reduced, CO-treated A-cluster (e.g., [162]) that is not formed in the independent CODH enzyme. Analysis of other states of the A-cluster is facilitated by the availability of purified  $\alpha$ -subunits, which contain only this metal site (e.g., [167,168]). On the basis of a variety of biochemical and spectroscopic results from such studies, the A-cluster was suggested to be a [4Fe-4S] center somehow coupled to a Ni ion. This proposal was confirmed by two X-ray crystallography studies of M. thermoacetica ACDS [165,215] that reveal two versions of the A-cluster structure shown in Fig. 1D. As expected, this site does possess a typical [4Fe-4S] cluster bridged to Ni; however, the ligands to the Ni and the identity of the bridge were unanticipated. The Ni is bound in a Cys-Gly-Cys motif, with two thiolate ligands and two peptide backbone amide interactions making up the four-coordinate planar geometry. According to one published structure [165], a four-coordinate copper atom connects the Ni to the [4Fe-4S] cluster. This Cu(I) shares one thiol ligand with a cubane iron atom and shares both thiols that bind the Ni. An unidentified fourth ligand (labeled AYT in the figure) may be an acetyl group or CO occupying two different geometries. On the basis of this remarkable structure, the authors propose an intriguing catalytic mechanism in which CO (generated at the CODH end of the tunnel) binds to Cu(I), a methyl group is transferred to Ni(II), methyl migration results in an acetyl group bound to Cu, and thiolysis by CoA provides acetyl-CoA [165]. The authors suggest that Cu is likely to remain Cu(I) throughout the catalytic cycle, consistent with the lack of any Cu(II) EPR signal associated with the active site. The redox states of other components of the active site during the proposed catalytic cycle are unclear and do not appear to completely reconcile with the known Mössbauer and UV/visible spectroscopic properties of the enzyme. Furthermore, the proposed structure of the A-cluster is inconsistent with the 1,10-phenanthroline-labile nature of the unique Ni site [166]. Finally, a comparison of metal content versus activity and NiFeC signal intensity [216] does not provide a convincing case for the essentiality of Cu in this metal site.

The second ACDS crystal structure [215] provides several additional structural details and, of greatest interest, shows the bridging metal to be Zn or a second atom of Ni. The authors suggest that active enzyme contains a [4Fe-4S]-Ni-Ni cluster (i.e., with the cyan Cu of Fig. 1D replaced by Ni). Such a situation would more readily account for the labile Ni site of the enzyme [166]. Support for a dinickel A-cluster structure is derived from studies of recombinant M. thermophila β-subunit (homologous to the  $\alpha$ -subunit of the *M. thermoacetica* enzyme) [169]. The overproduced protein contains an Fe-S cluster, but is inactive and has no Ni or Cu. Simple addition of Ni ions leads to production of high levels of ACDS activity and the ability to form the NiFeC signal. Significantly, the ratio of Fe to Ni in the activated protein is 2:1, consistent with two Ni in the A-cluster. On the basis of the structure of the acetogen enzyme, Darnault et al. [215] propose a novel mechanism for this cluster in which the [4Fe-4S] and distal Ni modulate the reactivity of the central Ni via their shared Cys ligands, allowing redox cycling between the Ni(0) and Ni(II) states. Further studies are required to test this provocative hypothesis.

Few details are known concerning the mechanism by which the two Ni-containing active sites are assembled into the dual-activity ACDS proteins. On the basis of results from studies of R. rubrum CODH, it is reasonable to assume that a minimum of two accessory proteins is necessary just to assemble the CODH-type [1Ni-4Fe-4S] or [1Ni-4Fe-5S] site. Additional accessory proteins may be required to synthesize the [4Fe-4S]-Cu-Ni or [4Fe-4S]-Ni-Ni acetyl-CoA site. For example, if Cu is present in the active enzyme one may expect to require a specific Cu metallochaperone since free Cu ions are thought to not exist inside cells [170]. Consistent with the involvement of accessory proteins, ACDS gene clusters of both methanogens and acetogens contain non-subunit open reading frames, one of which encodes a CooC-like protein (e.g., [171,172]). Trace levels of ATPase activity are associated with the purified *M. thermoacetica* homologue (AcsF) [171]. On the other hand, heterologous expression in E. coli of the M. thermoacetica genes encoding only the enzyme subunits (acsAB) yields protein with CODH activity [173], suggesting that the C-cluster self-assembles or the required accessory proteins are available in E. coli. Even more surprising, overnight incubation of the purified protein with 0.2 mM Ni ions yields sample exhibiting the NiFeC EPR signal and possessing CO exchange activity [173]. These results are reminiscent of those mentioned above for activation of the methanogen  $\beta$ -subunit [169]. The ability to activate purified recombinant acetogen and methanogen proteins simply by incubating with Ni ions does not rule out a requirement for accessory proteins for in vivo activation. In particular, these cell-free activation events are very slow and need high concentrations of the metal ion that would not occur in the cell. Further studies are needed to clarify the requirements and mechanism of active site maturation in these proteins.

#### 4.5. Methyl-coenzyme M reductase

An estimated  $10^9$  tons of methane per year are generated in anaerobic habitats by methanogenic archaea growing on simple precursors such as acetate, methanol, methylamines, formate, and carbon dioxide plus hydrogen gas. As already described, utilization of both acetate and hydrogen occur by Ni-dependent processes (see Sections 4.4 and 4.2). This section focuses on an additional Ni-dependent enzyme, methyl-coenzyme M reductase, of central metabolic importance to all of these cells. The requirement for Ni associated with this enzyme explains the longknown Ni growth dependence of methanogens (e.g., [174]).

Methyl–coenzyme M reductase catalyzes the final step in methane formation, illustrated in Eq. 6. Two substrates are required by the enzyme, methyl–*S*–coenzyme M (CH<sub>3</sub>-S-CoM) and *N*-7-mercaptoheptanoylthreonine phosphate or coenzyme B (CoB-SH). CH<sub>3</sub>-S-CoM is formed by methylation of coenzyme M (HS-CoM, 2-thioethanesulfonate) using a variety of pathways to convert the diverse growth nutrients mentioned above to the bound methyl group. The enzyme converts the two substrates into methane plus the mixed disulfide, CoB-S-S-CoM, which is subsequently reduced by a separate heterodisulfide reductase in an energy-generating step (see [175] for an overview of methanogenesis biochemistry). Methyl–coenzyme M reductase and its Ni-containing cofactor,  $F_{430}$ , have been extensively characterized.

X-ray crystal structures have been obtained for methylcoenzyme M reductases from *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain Marburg), *M. barkeri*, and *Methanopyrus kandleri* [176–178]. In each case, the enzyme possesses an  $\alpha_2\beta_2\gamma_2$  structure ( $\alpha \sim 60$  kDa,  $\beta \sim 46$  kDa,  $\gamma \sim 28$  kDa) containing two active sites, each with a Ni-containing tet-



rapyrrole (Fig. 1E). The cofactor, designated F<sub>430</sub> on the basis of its 430-nm color after purification in its Ni(II) form, is tightly but non-covalently bound to the protein. It is deeply buried in the protein, but connected to the surface by a 30-A channel through which CH<sub>3</sub>-S-CoM must enter and which subsequently is filled by CoB-SH binding. The Ni of F<sub>430</sub> is bound by the four pyrrole nitrogens and a glutamine axial ligand, leaving a sixth coordination site available for interaction with CH<sub>3</sub>-S-CoM. Within the active enzyme, the metal redox state is Ni(I) resulting in cofactor absorptions at 386 and 750 nm [179]. On the basis of structural [176-178] and spectroscopic (e.g., [180–184]) studies of the enzyme in its various states, insight into the enzyme mechanism is beginning to emerge. Significantly, a key activation step involves the two-electron reduction of a C=N double bond within the tetrapyrrole ring according to resonance Raman vibrations [184]. This finding explains the anomalous results from prior activation studies that had demonstrated a required reductive step to convert one Ni(I) state (known as ox1) to a distinct state (red1) also containing Ni(I). X-ray absorption spectroscopy provides evidence that ox1 to red1 conversion is accompanied by dissociation of an axial thiolate ligand [184,185]. Binding of CH<sub>3</sub>-S-CoM precedes binding of CoB-SH, then nucleophilic attack by Ni(I)-F<sub>430</sub> on CH<sub>3</sub>-S-CoM likely leads to a CH<sub>3</sub>-Ni(III)-F<sub>430</sub> intermediate and CoM-SH. The CoM-SH probably is oxidized to the thiyl radical as CH<sub>3</sub>-Ni(II)-F<sub>430</sub> forms, with the latter species undergoing protonolysis to yield methane and Ni(II)-F<sub>430</sub>. The thiyl radical may react with the CoB thiolate to form the heterodisulfide anion radical that reduces the tetrapyrrole cofactor to the Ni(I) form, and yielding the final mixed disulfide.

Several post-translational modifications are found in methyl-coenzyme M reductase. In particular, 1-N-methyl-His, 5-methyl-Arg, 2-methyl-Gln, S-methyl-Cys, and Gly with sulfur substituted for the carbonyl oxygen are found in the *M. marburgensis* enzyme [176] and all but the modified Gln in the *M. barkeri* protein [177]. The methylations are not side products arising during catalysis, but appear to be formed by specific S-adenosylmethioninedependent enzymes [186]. The mechanism for forming the thio-Gly is unknown, but an intriguing possibility for its function is to act as an electron relay during catalysis. Many open questions remain to be answered concerning the synthesis of CH<sub>3</sub>-S-CoM reductase. For example, the timing of the chemical modifications and  $F_{430}$  insertion into the structure during protein folding is unknown. In addition, the identities of the modification enzymes and possible chaperones involved in this process are not identified. Clearly, this fascinating enzyme will continue to stimulate further studies.

Another topic of related interest centers on the biosynthetic pathway of the unique Ni-containing cofactor F430 [187]. Early labeling studies revealed that 5-aminolevulinic acid, a uroporphyrinogen III precursor, is incorporated into  $F_{430}$  and subsequent efforts demonstrated that this compound is an intermediate (e.g., [188]). Hemes, sirohemes, and corrinoids are all derived from uroporphyrinogen III, but only sirohemes and corrinoids are formed after conversion to dihydrosirohydrochlorin. The latter compound also serves as an intermediate in formation of F<sub>430</sub> on the basis of labeling studies with sirohydrochlorin [189]. Additional steps needed to convert this intermediate to F<sub>430</sub> include amidation of the acetates on two rings, reduction of two double bonds, cyclization of an acetamide to form the five-membered ring, cyclization of a propionate to form the six-membered ring, and insertion of Ni. The order of these steps and the enzymes involved in these processes are completely unknown. The enzymes ferrochelatase, Co-chelatase, and Mg-chelatase are known to insert the appropriate metals into hemes, corrinoids, and chlorophylls [190-192]; thus, it is likely that a Nispecific enzyme is required for inserting nickel ion.

#### 4.6. Ni-dependent superoxide dismutase

SODs function to protect cells from the cytoxicity of superoxide radical anion by carrying out the reaction illustrated in Eq. 7. Several forms of the enzyme have been isolated including those containing a Cu,Zn-dinuclear center and mononuclear Fe or Mn sites. Attesting to its physiological importance, multiple forms of the enzyme often are observed to occur in a single microorganism. In addition to the Cu,Zn-SOD, Fe-SOD, and Mn-SOD species, a Ni-containing form of the enzyme was identified in several strains of *Streptomyces* in 1996 [38–40]. Although Fe- and Mn-containing forms of the enzyme were also reported for particular strains [39,40], all clinical and soil isolates of *Streptomyces* possess the cytoplasmic Ni-containing enzyme [193]. The *sodN* gene encoding Ni-SOD was cloned from *S. coelicolor* [41] and *Streptomyces seoulensis* [194], and the sequences were found to be unrelated to those of other types of SODs. Initial studies reported that the enzyme is a homotetramer of  $\sim$  13-kDa subunits with nearly 1 Ni/subunit [38–40]; however, investigation of the crystallized protein suggests a hexameric nature of the protein (interpretation of the 1.68-A resolution structure is in progress) [195]. Biophysical studies reveal that as-isolated protein contains Ni(III) [38,39] coordinated by three sulfur atoms and two nitrogen/oxygen atoms [194]. Since the enzyme contains only two cysteine residues (found in a CxxxC motif), a mononuclear site in the enzyme would need to possess methionine ligation. An alternative interpretation is that a dinuclear site is present with the cysteines from each pair of subunits coordinating the metallocenter in a bridging geometry. This model is supported by the observed 0.5 electron spins/monomer quantitation of Ni(III) obtained by EPR measurements [194]. The forthcoming crystal structure will allow resolution of these competing models. No studies have yet addressed the mechanism by which Streptomyces species are able to selectively incorporate Ni into this unique enzyme.

$$2O_{2}^{\bullet-} + 2H^{+} \to H_{2}O_{2} + O_{2}$$
(7)

#### 4.7. Ni-dependent glyoxylase

Like SOD, glyoxylase is another enzyme involved in cellular protection; however, in this case the toxic species is methylglyoxal rather than a reactive oxygen species. Several enzymatic processes produce methylglyoxal including a minor side reaction of triosephosphate isomerase when using its normal substrates dihydroxyacetone phosphate and glyceraldehyde. Because these glycolysis intermediates and triosephosphate isomerase are so abundant in cells, significant amounts of methylglyoxal are generated and react to form covalent adducts of DNA and proteins. One mechanism to remove this reactive compound involves the two-component glyoxylase system, illustrated in Eqs. 8 and 9. Glutathione (G-SH) reacts with methylglyoxal in a non-enzymatic reaction to form the hemithioacetal substrate of glyoxylase I (Glx I). This enzyme carries out an isomerization reaction resulting in formation of S-D-lactoylglutathione (Eq. 8). The product of Glx I is the substrate for glyoxylase II (Glx II) that hydrolyzes it to form lactate (Eq. 9). Glx I from yeast and humans is well characterized and known to be a Zn-dependent enzyme. Surprisingly, the E. coli enzyme has a preference for Ni despite a high degree of sequence identity to these other enzymes [196]. The crystal structures of the active Ni enzyme and active Co- and Cd-substituted E. coli proteins each show octahedral metal coordination (see Fig. 1F for the Ni-bound structure), analogous to the situation for Zn in the human enzyme, whereas the inactive Zn-containing E. coli protein has a five-coordinate metal site [197]. X-ray absorption studies of enzyme-product and enzyme-inhibitor complexes [198] provide evidence consistent with an enzyme mechanism involving proton transfers between substrate and metalcoordinated solvent molecules, rather than direct coordination of substrate to the metal. On the basis of detailed sequence comparison of metal-binding residues, several pathogenic microorganisms including Y. pestis also are likely to possess Ni-dependent Glx I species [199]. Similar to the situation for Ni-dependent SOD, the mechanism of Ni incorporation into Glx I is unknown.

#### 4.8. Aci-reductone dioxygenase

Many microorganisms recycle methylthioadenosine, produced during polyamine biosynthesis from S-adenosylmethionine, back to the original amino acid by using the methionine salvage pathway. A key intermediate of this pathway is 1,2-dihydroxy-3-keto-5-methylthiopentane, also called aci-reductone. Investigations using Klebsiella pneumoniae reveal that aci-reductone is oxidized to two different sets of products (Fig. 5). In the productive case, a dioxygenase activity produces formic acid plus the  $\alpha$ -ketoacid precursor of methionine that is readily transaminated to provide the free amino acid. In addition, a second, non-productive dioxygenase activity converts the intermediate to formate, carbon monoxide, and methylthiopropionate. Amazingly, these activities belong to the same protein, but result from differences in metal content [200]. Using either the K. pneumoniae enzyme or recombinant protein produced in E. coli, the productive activity is seen with an Fe-containing protein and the non-productive activity is associated with protein containing Ni. By incubating either of the enzyme species with chelators and then adding the opposite metal ion, the activities are in-





Fig. 5. Aci-reductone dioxygenase reactions. The same protein carries out two distinct reactions depending on whether Fe or Ni is bound.

terconverted. This is the only known example of a Ni-dependent oxygenase activity. Mechanistic studies suggest that the identity of the metal ion dictates the site of attack by a putative peroxide anion generated during the reaction [201]. Biophysical studies demonstrate that the as-isolated Ni-containing enzyme coordinates the metal via three histidine side chains and three other nitrogen/oxygen donors [202]. Addition of substrate leads to displacement of one histidine and one other ligand, thus providing sites for binding of the adjacent hydroxyl groups of the substrate. The solution structure of Ni-bound aci-reductone dioxygenase was determined by NMR methods [203]. Unfortunately, the paramagnetic Ni broadens the resonance lines for all protons located within  $\sim 8$  Å of the metal ion, thus preventing structural characterization of the region immediately around the active site. On the basis of homology modeling to members of the cupin family, a reasonable structure of the active site region was determined (Fig. 1G). This structure is known to be incorrect in one respect; namely, NMR measurements indicate that one of the imidazoles actually coordinates the metal using its N $\delta$ 1 atom rather than the NE2 atoms as shown. The in vivo significance of the Ni-promoted reaction and the cellular mechanism of incorporating Ni into the enzyme are unknown.

#### 4.9. Other Ni enzymes

In unpublished studies (H. Ewen and T. Jahns, personal communication), methylenediurease was found to be a Nidependent enzyme. Methyleneureas, or ureaforms, are condensation products of urea plus formaldehyde [general formula H<sub>2</sub>N-(CO-NH-CH<sub>2</sub>-NH)<sub>i</sub>-CO-NH<sub>2</sub>] and are used as slow-release fertilizers [204]. An enzyme capable of degrading methylenediurea (i.e., n = 1 in the above formula) was isolated from a Burkholderia species and shown to possess stoichiometric amounts of bound Ni. Significantly, the methylenediurease activity was resolved by anion exchange chromatography from urease activity of the same microorganism, and each enzyme was specific toward its own substrate. Methylenediurease contains two subunits, the largest of which (according to DNA accession number AY194235) is clearly related to urease and includes residues corresponding to all of the ligands of the urease dinuclear Ni active site. Further studies are required to characterize the structural basis for the unique specificity, to define the mechanism of this enzyme, and to examine whether accessory proteins are required for its activation (or whether the urease accessory proteins may have dual roles and also activate this enzyme).

In addition to the Ni-dependent enzymes described above, many other metalloenzymes can function with Ni ions replacing the native Zn or other metal ions. Dissociation of a weakly bound metal ion, inadequate provision of the required metal ion in the medium, or overwhelming of the metal-delivery system for proteins that are highly expressed in the cell can all lead to the cellular production of apoproteins. The interaction of these proteins with added Ni can lead to the incorrect interpretation of Nidependent enzyme activity. Thus, great care must be exercised when drawing conclusions from such studies. As an illustration of an enzyme activity that was mis-identified as being Ni-specific, we cite E. coli peptide deformylase. The labile activity was found to be stabilized by addition of Ni, and a Ni-bound form of this recombinant enzyme was obtained and structurally characterized using Ni-containing buffers [205,206]. On the basis of these findings, peptide deformylase incorrectly was referred to as a Ni-dependent enzyme. Subsequent studies reveal that peptide deformylase actually contains Fe in its active state and that the oxidative lability of the ferrous ion during purification leads to rapid inactivation of this enzyme form [207–210].

#### 5. Concluding remarks

Since the initial observations of Ni-dependent growth of hydrogen-oxidizing bacteria in 1965 [1], the pace of research involving Ni uptake and utilization in microorganisms has continuously accelerated. Several Ni sensor/regulators have been identified, but their precise mechanisms for interacting with the metal ion and with their specific regulatory DNA sequences require further investigations. Two broad categories of uptake mechanisms, ABC-type transporters and Ni-specific permeases, have been identified in numerous microorganisms, but many molecular details about these systems remain to be elucidated. For example, the energetics used to drive the Ni permease systems are unknown and the residues conferring specificity are only beginning to be revealed. Progress in understanding the structures and mechanisms of the nine known Ni enzymes are well advanced, but gaps remain in our understanding of the roles of the metal ions in catalysis. Similarly, the maturation mechanisms of these enzymes are starting to be understood, but basic questions remain concerning the availability of Ni in the cell, the basis of specificity, and the roles of the accessory proteins. Given the short time span since the first Ni enzyme was identified (urease was shown to contain Ni in 1975 [73]), it would

not be surprising if additional examples of Ni-enzymes are discovered.

#### Acknowledgements

Studies in the authors' laboratory are supported by the National Institutes of Health Grant DK45686 (to R.P.H.).

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