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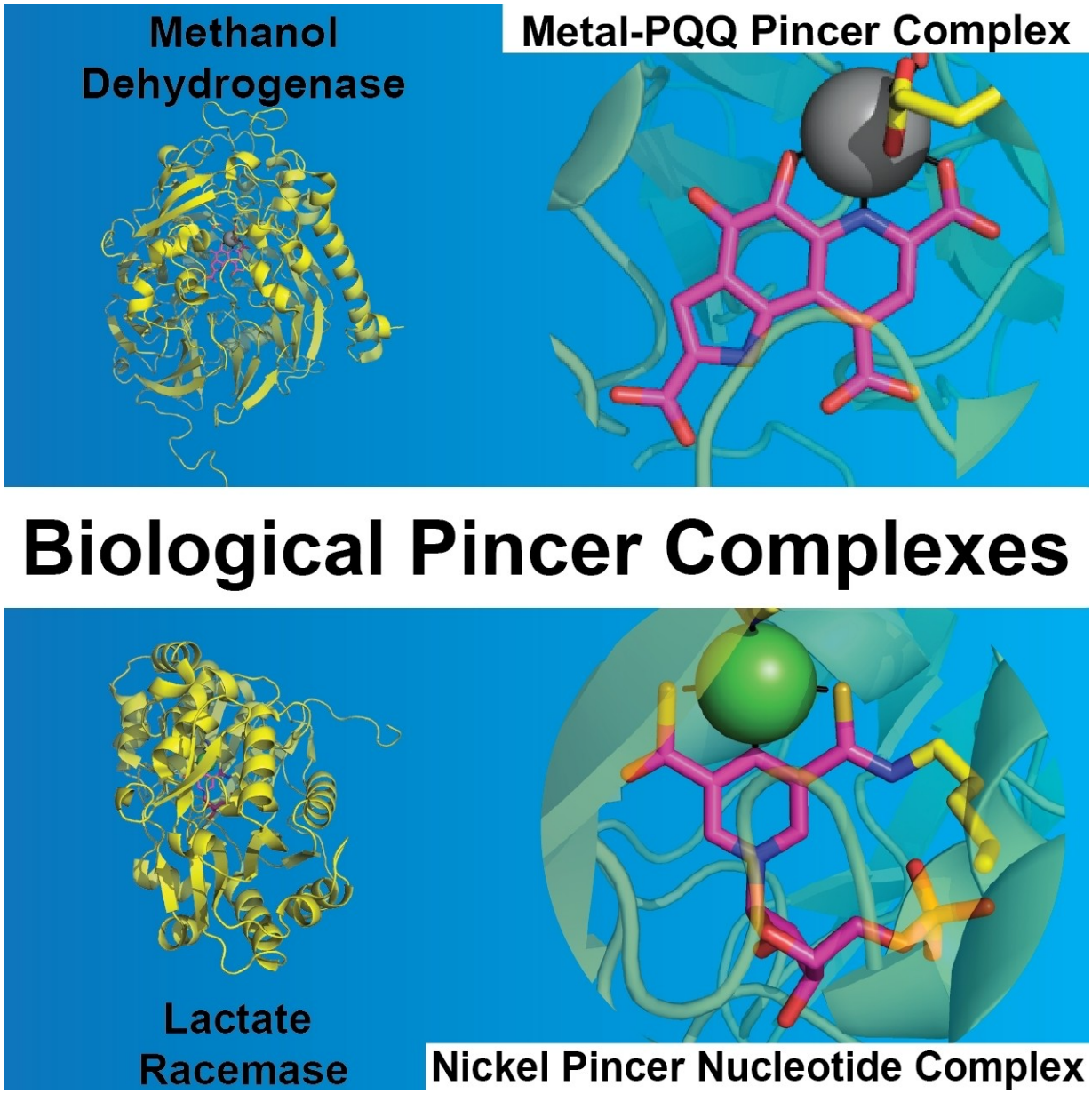
Biological Pincer Complexes

Jorge L. Nevarez+,[a] Aiko Turmo+,[b] Jian Hu,*[a, b] and Robert P. Hausinger*[b, c]



At least two types of pincer complexes are known to exist in biology. A metal-pyrroloquinolone quinone (PQQ) cofactor was first identified in bacterial methanol dehydrogenase, and later also found in selected short-chain alcohol dehydrogenases of other microorganisms. The PQQ-associated metal can be calcium, magnesium, or a rare earth element depending on the enzyme sequence. Synthesis of this organic ligand requires a series of accessory proteins acting on a small peptide, PqqA. Binding of metal to PQQ yields an ONO-type pincer complex. More recently, a nickel-pincer nucleotide (NPN) cofactor was discovered in lactate racemase, LarA. This cofactor derives from nicotinic acid adenine dinucleotide via action of a carboxylase/hydrolase, sulfur transferase, and nickel insertase, resulting in an SCS-type pincer complex. The NPN cofactor likely occurs in selected other racemases and epimerases of bacteria, archaea, and a few eukaryotes.

1. Introduction

This special collection of articles on pincer chemistry and catalysis focuses primarily on the properties and reactivities of pincer complexes synthesized by inorganic chemists. In this contribution, we describe two types of pincer complexes identified in biological systems: the metal-pyrroloquinoline quinone (PQQ) cofactor, containing an ONO-type pincer ligand, found in particular dehydrogenases, and the nickel-pincer nucleotide (NPN) cofactor, with an SCS-type pincer ligand, of selected racemases and epimerases. Both biological cofactors possess a planar organic ligand that tri-coordinates a metal ion (Figure 1) – the hallmark of a pincer complex. The following sections briefly review the discovery of each cofactor, summarize their biosynthetic pathways, detail how they function in catalysis, and compare their properties to the synthetic systems.

2. Metal-PQQ complexes

A novel prosthetic group was found in glucose dehydrogenase from *Bacterium anitratum* in 1964,[1] and three years later was also reported to be present in methanol dehydrogenase of *Pseudomonas* sp. M27.[2] Using cell-free extracts of the facultative methylotroph *Pseudomonas* TP1, the cofactor was purified and structurally defined as PQQ by x-ray crystallography.[3] The crystal structures of methanol dehydrogenases from *Methylophilus methylotrophus* and *Methylophilus* W3A1 at 2.6 Å resolution provided the first glimpses of how PQQ binds to this enzyme's active site.[4] The cofactor is coplanar with a tryptophan residue and located in a funnel-shaped channel. Various residues interact with the three carboxyl groups of PQQ and an arginine residue is positioned near the quinone moiety. Some, but not all, PQQ-dependent enzymes also contain metal ions and other distinguishing features. For example, structural studies of methanol dehydrogenase from *Methylorubrum* (formerly *Methylobacterium*) *extorquens* culminated in a 1.2 Å resolution structure (PDB ID 1W6S) revealing a disulfide bridge nearby the cofactor and showing that PQQ binds a calcium ion using its O5, N6, and O7 atoms, with additional metal coordination by Glu177 (bidentate) and Asn261 side chains.[5] Asp303 is located near the calcium ion in this structure, and a similarly positioned aspartic acid serves as an additional metal ligand in Ca-PQQ methanol dehydrogenases from several other microorganisms including *M. methylotrophus* W3A1, *Hyphomicrobium denitrificans*, and *Methanococcus capsulatus* strain Bath.[6] In studies of methanol dehydrogenase from *Methylacidiphilum fumariolicum* SolV, the metal speciation of the PQQ pincer complex was expanded to include several rare earth elements (lanthanum, cerium, neodymium, praseodymium, samarium, europium, or gadolinium).[7] The structures of the cerium-PQQ and europium-PQQ methanol dehydrogenases were shown to be very similar to the earlier described Ca-PQQ enzymes with the lanthanides tricoordinated to PQQ, but with four side chain metal ligands: a glutamic acid, an asparagine, and two aspartic acids.[7–8] A lanthanide-PQQ cofactor also was structurally characterized from *Methylmicrobium buryatense* 5GB1C methanol dehydrogenase,[9] whereas a magnesium-PQQ cofactor was identified in the enzyme from *Methylophaga aminisulfidivorans*.[10] *M. extorquens* AM1 is notable in containing a Ca-PQQ methanol dehydrogenase (encoded by *mxaF*), two lanthanide-PQQ methanol dehydrogenases (encoded by *xoxF1* and *xoxF2*), and a lanthanide-PQQ ethanol dehydrogenase (encoded by *exaF*).[11] Structural studies of *M. extorquens* XoxF1 combined with mutagenesis, metal-binding, and activity assays of this protein and *M. extorquens* ExaF provide evidence that the fourth metal ligand, an aspartate residue, determines whether a lanthanide is bound and functionally active in these proteins.[12] Lanthanide-PQQ alcohol dehydrogenases are also found in *Pseudomonas putida* KT 2440.[13] Recent evidence suggests this microorganism possesses two dehydrogenases that convert the glycerol

[a] J. L. Nevarez,[+] Prof. J. Hu
Department of Chemistry
578 South Shaw Lane
Michigan State University
East Lansing
Michigan 48824 (USA)
E-mail: hujian1@msu.edu
[b] A. Turmo,[+] Prof. J. Hu, Prof. R. P. Hausinger
Department of Biochemistry and Molecular Biology
603 Wilson Road, Room 212
Michigan State University
East Lansing
Michigan 48824 (USA)
E-mail: hausinge@msu.edu
[c] Prof. R. P. Hausinger
Department of Microbiology and Molecular Genetics
567 Wilson Road
2215 Biomedical Physical Sciences
Michigan State University
East Lansing
Michigan 48824 (USA)
Homepage: https://twitter.com/msu_mmg
[+] These authors contributed equally to this work.

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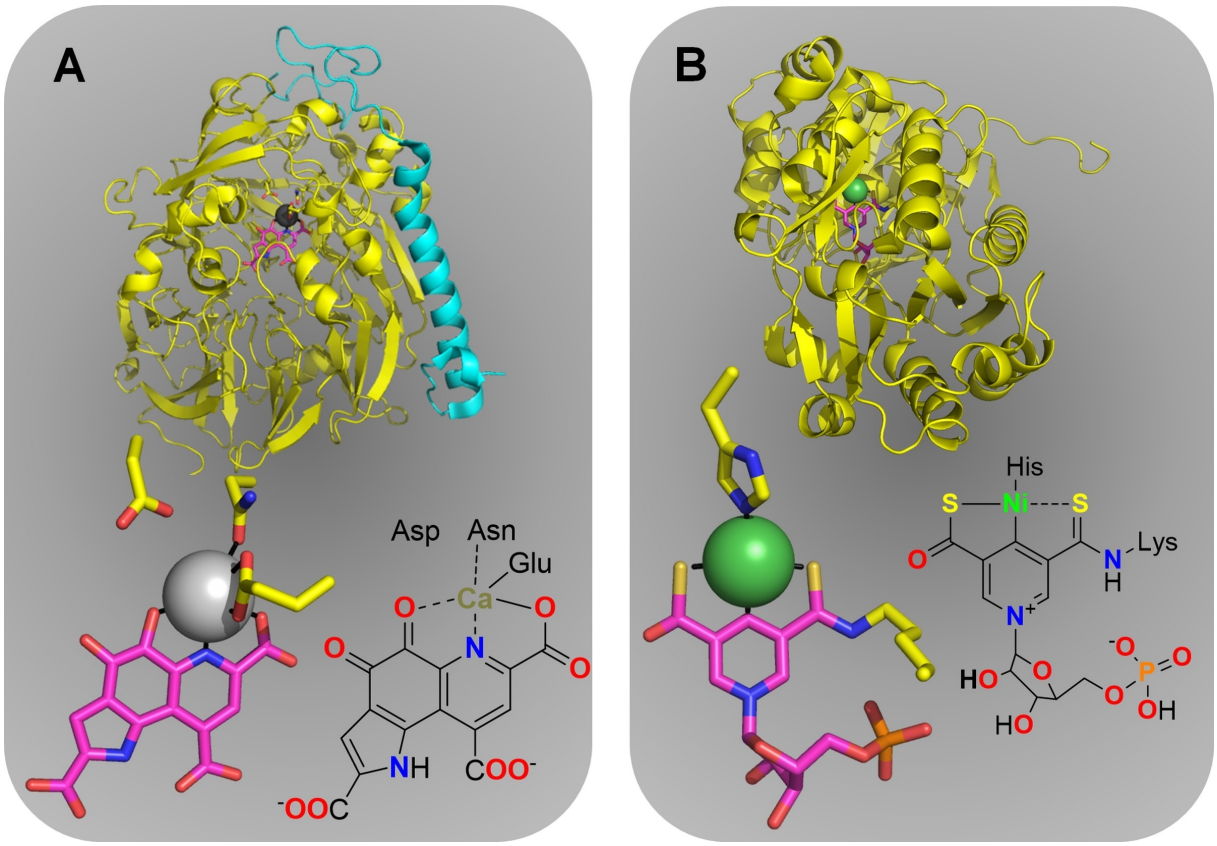
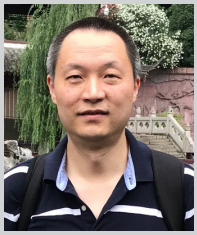


Figure 1. Biological pincer complexes. (A) Methanol dehydrogenase of *Methylorubrum extorquens* (PDB ID 1W6S) with its two protein subunits (yellow and cyan ribbons) shown in cartoon mode, PQQ in stick mode (magenta carbon atoms, blue nitrogen atoms, and red oxygen atoms), Glu177 (bidentate) and Asn261 metal-binding residues and the nearby Asp303 (a comparable residue is used as a metal ligand in other methanol dehydrogenases) in stick mode, and calcium as a grey sphere.[5c] A fourth metal ligand, an aspartic acid residue, is found in lanthanide-PQQ enzymes. The lower portion of the panel illustrates a close-up view of the Ca-PQQ complex and a ChemDraw depiction of the cofactor. (B) Lactate racemase of *Lactobacillus plantarum* (PDB ID 6C1W) with the protein shown as a yellow ribbon, Lys184 and His200 residues (yellow) as well as the NPN cofactor (with color as in panel A along with yellow sulfur, and orange phosphorus) as sticks, and nickel as a green sphere.[16] An expanded view of the Ni-NPN complex and a ChemDraw depiction are provided underneath.

substrate to glyceraldehyde; PedE using a calcium-PQQ enzyme and PedH using a lanthanide-PQQ version.[14] The physiological significance of lanthanide incorporation into the PQQ-containing enzymes in various microbes remains uncertain, but probably relates to the greater Lewis acidity of this metal when compared to calcium or magnesium. In addition to catalyzing

Jorge L. Nevarez earned his B.S. degree in Chemistry at Northern Illinois University in 2018. He is now a graduate student co-mentored by Drs. Hausinger and Hu. His current research focuses on elucidating the broader role of the nickel-pincer nucleotide cofactor in nature.

Aiko Turmo received B.S. degrees in Molecular Genetics and Genomics & Microbiology from Michigan State University in 2013. She was a research technician for four years, and is now a Ph.D. candidate in the Hausinger laboratory. Her current research focuses on the biochemical and structural characterization of the nickel insertase enzyme critical to the synthesis of the nickel-pincer nucleotide.



Jian Hu received his Ph.D. from Peking University Health Science Center, China, in 2004 and obtained postdoctoral training at Florida State University (2005-2007) and Yale School of Medicine (2007-2013). He joined Michigan State University in 2013 with appointments in Biochemistry & Molecular Biology and Chemistry. His research focuses on structural biology of metal transporters and metalloenzymes.

Robert P. Hausinger received his Ph.D. from the University of Minnesota (1982) and obtained postdoctoral training at M.I.T. (1982-1984). He is a University Distinguished Professor of Microbiology & Molecular Genetics and Biochemistry & Molecular Biology at Michigan State University. His research focuses on metallocenter biosynthesis and the catalytic mechanisms of metalloenzymes.

dehydrogenation of primary alcohols, some PQQ-containing enzymes oxidize formaldehyde and acetaldehyde to their respective acids.[1,15]

2.1 Biosynthesis of PQQ

The biosynthesis of PQQ (Scheme 1) is a topic of long-standing interest and requires the products of a multigene operon.[17] The tricyclic quinone structure of this cofactor derives from a small peptide named PqqA with a one-letter sequence of WKKPAFIDLRLGLEVTLYISR (for *Klebsiella pneumoniae*) containing conserved glutamic acid and tyrosine residues (underlined) that are used to form the PQQ structure.

PqqE, a radical *S*-adenosylmethionine (SAM)-dependent enzyme containing two [4Fe4S] clusters, catalyzes the formation of a C–C cross-link between C3 of the glutamic acid and the meta-position of tyrosine in the peptide while transforming SAM into methionine and 5′-deoxyadenosine ($AdoCH_3$) (Scheme 1).[18] This reaction is facilitated by the 10-kDa PqqD that forms a 1:1 complex with PqqE and may deliver PqqA to the catalyst.[19] The structure of a dimeric PqqD (PDB ID 3G2B) was reported for the protein from *Xanthomonas campestris* and shown to have a distinct saddle-type shape.[20]

A protease then trims off the extra residues from the crosslinked PqqA peptide (Scheme 1). In *Serratia marscescens*, this cleavage has been proposed to occur using a protein denoted PqqF.[21] The clamshell-shaped PqqF structure (PDB ID 5CIO) indicates it is a member of the inverzincin family with a zinc atom bound by two histidines and a glutamic acid (and an inhibitory histidine residue from the protein's His-tag).

An Fe-dependent hydroxylase, PqqB, converts the modified tyrosine into a hydroxyquinone (Scheme 1).[22] The structure of PqqB from *Pseudomonas putida* KT2440 was determined (PDB ID 6E13) and found to have a metallo β-lactase fold with two metal-binding sites per monomer; one site binds a structural zinc atom and the second binds iron using an aspartate and two histidine residues. The structure has zinc substituted for the catalytic iron atom and has the bound substrate analog 5-cysteinyl-3,4-dihydroxyphenylalanine. Two potential mechanisms of the enzyme were proposed, but further studies are needed to establish the steps of this chemical transformation.[22]

The product of PqqB, 3a-(2-amino-2-carboxyethyl)-4,5-dioxo-4,5,6,7,8,9-hexahydroquinoline-7,9-dicarboxylic acid (AHQQ), is converted to PQQ by PqqC, a seven-helix bundle protein (PDB ID 1OTW), that catalyzes an 8-electron oxidation without the use of an enzyme cofactor.[23] Three of these oxidation steps require dioxygen and produce hydrogen peroxide, but one oxidative reaction uses hydrogen peroxide that is reduced to two molecules of water (Scheme 1). No studies have examined the mechanism of metal binding to PQQ within the enzyme, but evidence suggests that the number and identity of the active site side chains determine which type of metal is selected.[12]

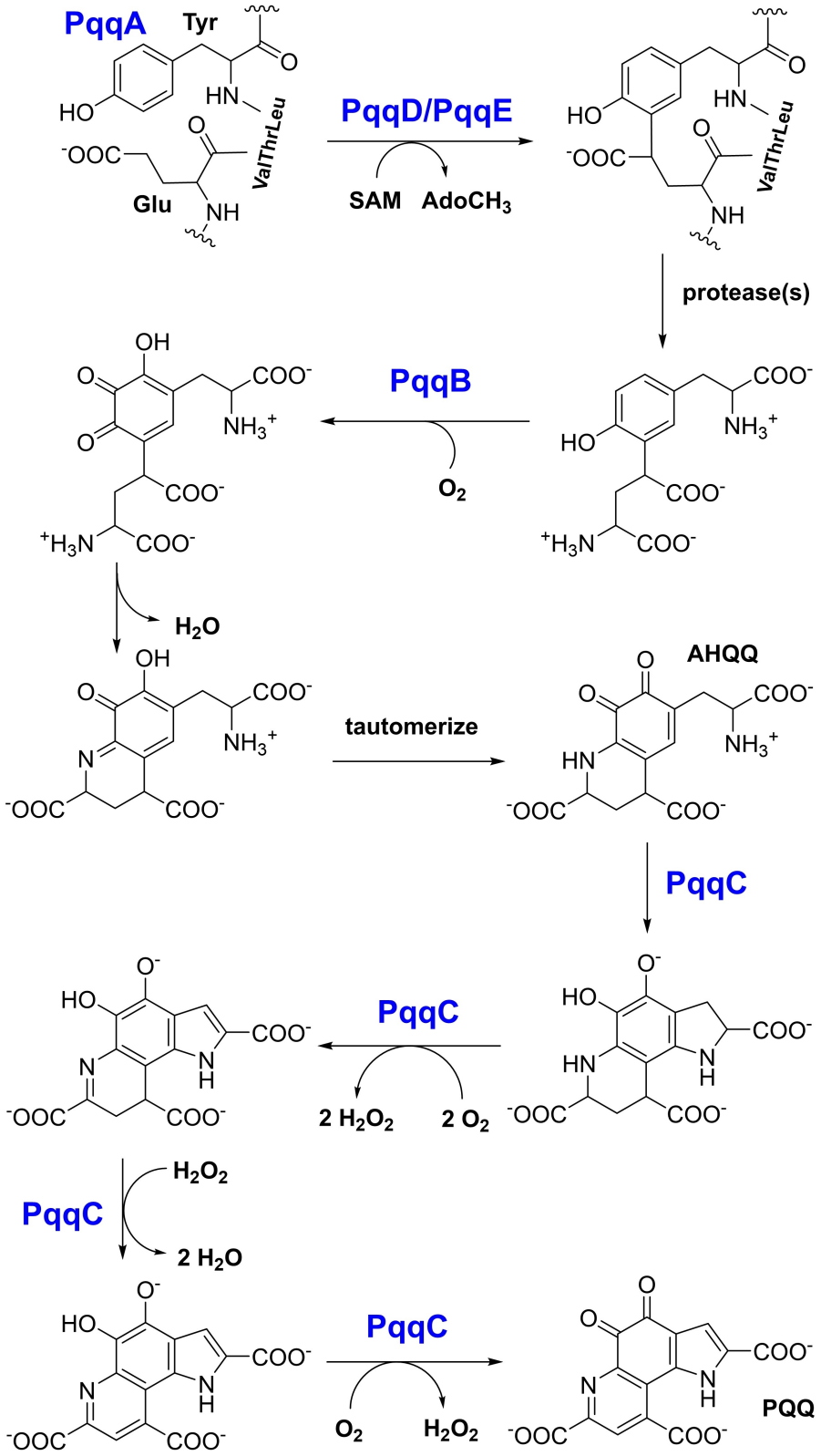
2.2. Mechanism of the metal-PQQ cofactor in alcohol dehydrogenation

Scheme 2 illustrates a proposed hydride transfer reaction mechanism of metal-PQQ dehydrogenases. A carboxylate group serves as a general base (B:) that deprotonates the substrate hydroxyl group, leading to the aldehyde formation and hydride transfer to C5 of PQQ. Subsequent proton transfer and tautomerization afford the reduced PQQ (a dihydroxyenol species), and two one-electron transfers to cytochrome c_L complete the cycle.[6b,24] When using an aldehyde as a substrate, the same sequence of reactions occur using the hydrated species (not shown). In these reactions, the metal serves as a Lewis acid, polarizing the C5–O5 bond and thus facilitating nucleophilic addition of hydride onto C5.

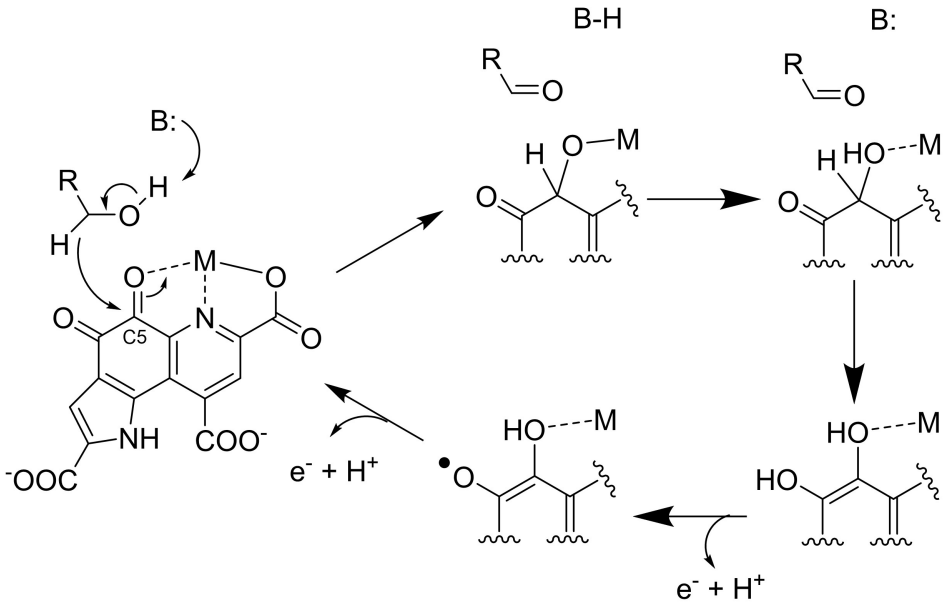
Metal-pincer complexes have long been known to catalyze dehydrogenation reactions.[25] It is worth noting that synthetic PQQ analogues exist and their abilities to dehydrogenate primary alcohols have been tested. An early example was the calcium complex of the 2,9-dimethyl ester of PQQ that catalyzes the dehydrogenation of benzyl alcohol (11 turnovers in 24 h).[26] This approach was extended to include a variety of calcium-PQQ model compounds that oxidize several types of alcohols.[27] Other PQQ-like models also have been investigated, including complexes with copper, iron, and other metals, but they are not described further because they are not biologically relevant. Of particular interest, a synthetic PQQ analogue denoted L_{QQ} (Scheme 3) was shown to form a complex with La and nitrate, $[La(L_{QQ})(NO_3)_3]$.[28] The X-ray crystal structure of $[La(L_{QQ})(NO_3)_3]$ indicates La coordination by the pyridyl nitrogen, a quinone oxygen, and a nitrogen linking atom of L_{QQ}, akin to the PQQ-metal coordination in dehydrogenase active sites, with two amide oxygen atoms in the pendant group substituting for protein side chains. Furthermore, the $[La(L_{QQ})(NO_3)_3]$ complex is capable of catalyzing the dehydrogenation of 4-methylbenzyl alcohol to give 4-methylbenzaldehyde (30% yield in 24 h, 67% yield in 3 days). This reaction is accelerated in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (63% yield in <10 min) or 2,6-lutidine (37% yield in <24 h). Coupling these reagents with an oxidant ($[Fc][PF_6]$) led to 84% yield in 21 h, accounting for 17 turnovers of the catalyst.[28] Density functional theory (DFT) calculations for this complex support a hydride transfer mechanism over a competing proposal involving an addition-elimination mechanism. The demonstration of function using such synthetic models is exciting, but the catalytic abilities of these complexes pale in comparison to the enzyme case.

3. NPN cofactor

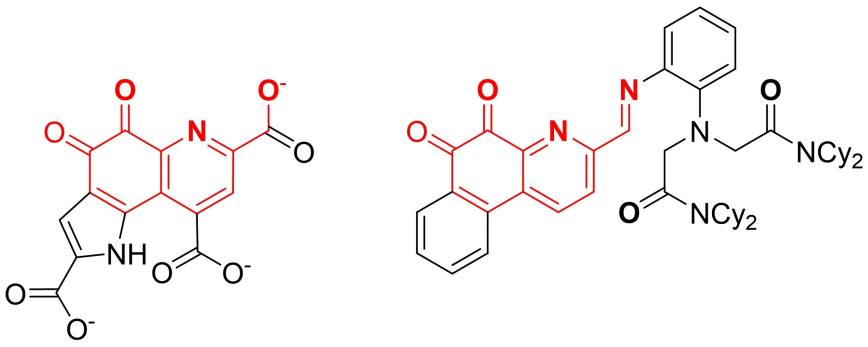
The structure of lactate racemase (LarA) from *L. plantarum* with its NPN cofactor (Figure 1B)[16] raises questions, both about its synthesis and its role. These topics are addressed in the following two sections.



Scheme 1. Biosynthetic pathway for PQQ. A five-residue portion of PqqA (Glu-Val-Thr-Leu-Tyr) is depicted. The radical SAM enzyme PqqE forms a C–C crosslink between the Glu and Tyr residues with the assistance of PqqD. Protease activity (perhaps involving PqqF) trims the compound to just the crosslinked dimer, which is oxidized by the iron-containing enzyme PqqB. Imine formation followed by tautomerization yields the AHQQ intermediate. PqqC then catalyzes four sequential two-electron oxidation steps, three of which use O_2 and form H_2O_2 whereas one step uses H_2O_2 and forms two molecules of water.



Scheme 2. Role of the metal-PQQ pincer cofactor in the reaction mechanism of alcohol dehydrogenases.



Scheme 3. Comparison of the structures for PQQ and a chemical model, L_{QQ}. The regions of similarity are indicated in red and the atoms that coordinate the metal are shown in bold. Cy = cyclohexyl.

3.1. Biosynthesis of the NPN cofactor

Genetic studies identified three genes required for synthesis of active lactate racemase (*larB*, *larC*, and *larE*) that are co-localized and co-regulated with *larA* in *L. plantarum*.[29] Subsequent investigations revealed the pathway by which the corresponding gene products function in the biosynthesis of the novel SCS-type nickel-pincer complex (Figure 2).

The NPN cofactor is derived from nicotinic acid adenine dinucleotide (NaAD) with LarB catalyzing the first steps: carboxylation on C5 of the pyridinium ring while hydrolyzing the phosphoanhydride with release of AMP to form pyridinium-3,5-dicarboxylic acid mononucleotide (P2CMN).[30] It is plausible that carboxylation occurs first, followed by hydrolysis of the dicarboxylated dinucleotide to promote P2CMN release. Conversely, direct hydrolysis of NaAD with release of nicotinamide mononucleotide and AMP also occurs, suggesting LarB may hydrolyze NaAD prior to carboxylation. No external energy source is required for this reaction and it has been speculated that the energy released from the hydrolysis of NaAD is used for the carboxylation reaction.[30] Further studies are needed to establish whether LarB uses CO_2 or bicarbonate as the substrate, to identify functions of highly conserved residues in the protein, and to define the structural basis of the carboxylation and hydrolysis reactions.

In the second stage of the NPN biosynthetic pathway, two molecules of LarE each sacrifice a cysteine side chain sulfur atom, forming dehydroalanine residues (Dha), while sequentially converting the P2CMN carboxyl groups into thioacids, thus forming pyridinium-3,5-dithiocarboxylic acid mononucleotide (P2TMN).[30–31] On the basis of structural and mechanistic studies, each sulfur transfer reaction involves (i) ATP-dependent activation of a substrate carboxyl group by adenylylation with the release of pyrophosphate, (ii) cysteine residue attack on the activated substrate to form a thioester with the release of AMP, (iii) deprotonation of the cysteine Cα position, and (iv) sulfur

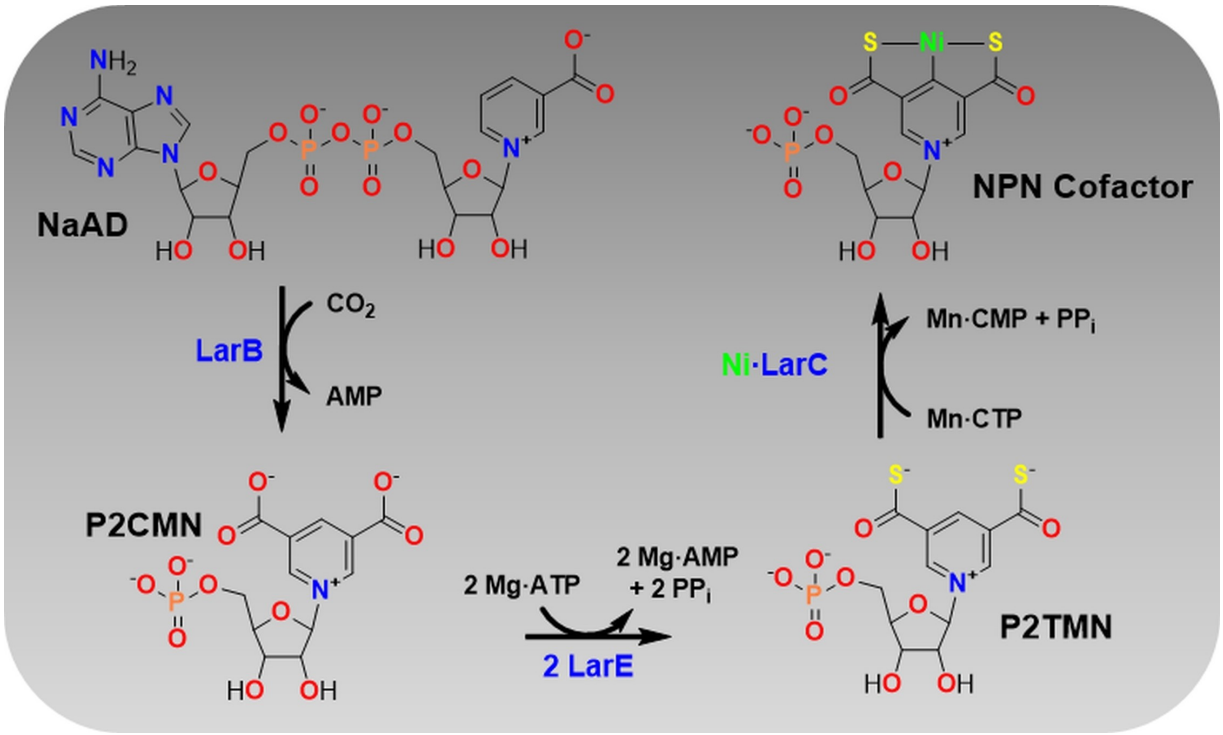


Figure 2. Biosynthesis of the NPN cofactor in *L. plantarum*. LarB initiates the pathway by catalyzing the carboxylation/hydrolysis of NaAD to produce P2CMN. Two LarE subunits each sacrifice a cysteine residue sulfur atom while catalyzing an ATP-dependent reaction to generate P2TMN. Finally, LarC inserts nickel by a CTP-dependent reaction to form the NPN cofactor. CTP = cytidine triphosphate, NaAD = nicotinic acid adenine dinucleotide, P2CMN = pyridinium-3,5-dicarboxylic acid mononucleotide, P2TMN = pyridinium-3,5-dithiocarboxylic acid mononucleotide, NPN = nickel-pincer nucleotide.

transfer to form the product thioacid. This type of sacrificial sulfur transfer resulting in a Dha residue is known to occur in only one other enzyme, thiamine thiazole synthase from *Saccharomyces cerevisiae*.[32] The Dha-containing form of LarE is capable of being recycled *in vitro* by incubation with the persulfide of coenzyme A (CoA) followed by addition of a reductant.[33] In this recovery reaction, the highly nucleophilic persulfide adds to the Dha residue yielding a CoA-LarE mixed disulfide that subsequently undergoes reduction. It is unclear whether Dha recycling is physiologically relevant; however, CoA binds to and stabilizes LarE.[30,33] In addition to the LarE adducts with P2CMN and pyridinium-3-carboxy-5-thiocarboxylic acid mononucleotide formed during the sulfur transfer reactions, there is evidence for a LarE adduct of NPN, suggesting that nickel can insert into P2TMN while covalently bound to LarE.[33] This result explains why large amounts of isolated *L. plantarum* LarE, when purified from cells that co-produce LarB and LarC, can activate LarA apoenzyme.[29]

In the terminal step of NPN cofactor biosynthesis, LarC installs nickel by forming new nickel-carbon and nickel-sulfur sigma bonds.[34] This capability makes LarC the first enzyme identified to catalyze a cyclometallation reaction.[35] The molecular mechanism of the enzymatic reaction is unknown; however, activity assays demonstrate that LarC is a cytidine triphosphate (CTP)-dependent enzyme.[34] A novel nucleotide-binding site was identified by x-ray crystallographic analysis of the protein C-terminal domain in complex with CTP and by site-directed mutagenesis studies that showed the CTP-binding residues are required.[34] LarC hydrolyzes CTP to form CMP, implying substrate activation by cytidylylation; however, nucleotide hydrolysis could alternatively relate to a protein conformational change. Of added interest, LarC appears to function stoichiometrically rather than catalytically; i.e., this is a single-turnover enzyme.[34]

A possible function of CTP in the LarC-catalyzed nickel insertion step is derived from studies involving the molybdenum cofactor biosynthesis pathway.[36] The molybdenum insertase of eukaryotes contains two functional domains: the G domain that adenylylates the molybdopterin (MPT) cofactor precursor and the E domain that inserts molybdenum while hydrolyzing the adenylylated precursor.[37] A high-resolution crystal structure of a plant molybdenum insertase E domain demonstrated that the AMP-MPT adduct serves to anchor the MPT substrate properly to coordinate insertion of the molybdenum.[38] In a similar manner, LarC might cytidylylate P2TMN to properly position the substrate and then hydrolyze the CMP-NPN adduct after inserting the nickel (Figure 3).

Once synthesized, the NPN cofactor incorporates into the lactate racemase protein. In the case of *L. plantarum*, one of the thioacids of the cofactor covalently links to a lysine residue as a thioamide.[16] By contrast, in *Thermoanaerobacter thermosaccharolyticum* lactate racemase the cofactor binds non-covalently to the protein.[29] The apoprotein of this species has

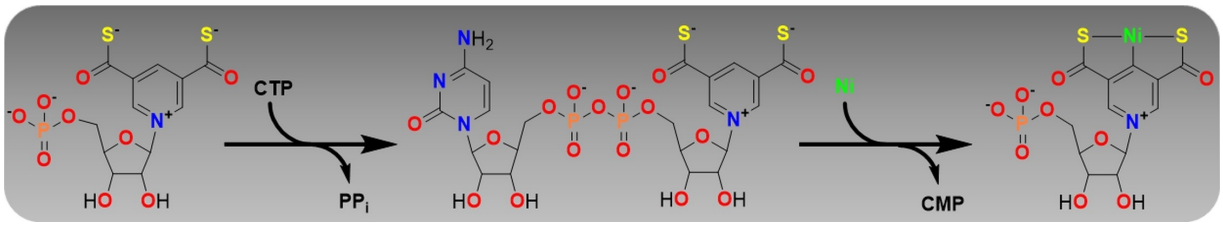


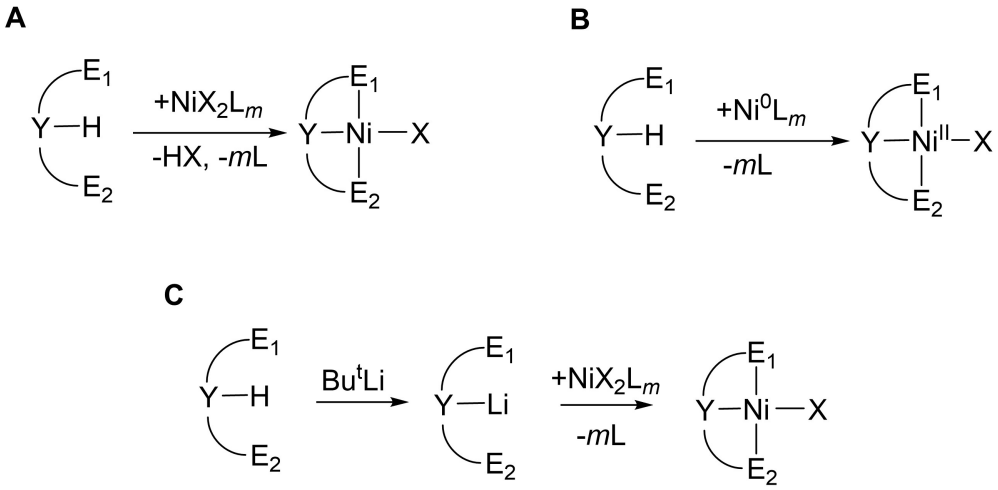
Figure 3. One possible role for CTP in the LarC-catalyzed nickel insertion step is to serve as a handle to allow for more precise positioning of the pincer ligand at the metal-binding site.

been useful for monitoring NPN cofactor biosynthesis based on its ability to rapidly confer lactate racemase activity.[30,34]

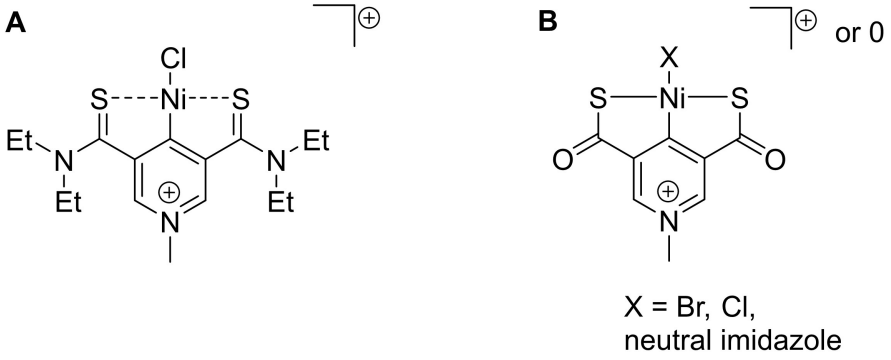
Characterization of the NPN biosynthesis pathway has focused on enzymes from *L. plantarum*; however, an analysis of over 1,000 bacterial and archaeal genomes indicate about 9% contain genes that may encode LarA and the NPN biosynthetic proteins.[29] Investigating selected homologs might lead to the discovery of alternative enzymes with distinct catalytic properties for generating the NPN cofactor. As an example, homology models of some LarE homologs are consistent with a tri-cysteine motif that could possibly bind an iron-sulfur cluster.[31] Transfer of an extra-cluster sulfur atom to substrate potentially could be energetically less costly than providing sulfur from a cysteine residue. Furthermore, genome analyses have identified natural fusions of some of the biosynthetic enzymes, possibly allowing for channeling of the pathway in Nature. An additional ~15% of the same list of genomes lack a homolog of the *larA* gene, but contain homologs to *larB*, *larC*, and *larE*. This finding suggests that the NPN cofactor is synthesized for purposes other than lactate racemization. Furthermore, some organisms have multiple paralogs of *larA*, again consistent with alternative roles for the cofactor.[29] We discuss the roles of NPN in lactate racemase and other reactions in the next section.

Before leaving this section on NPN cofactor biosynthesis, it is informative to compare the nickel insertion reaction catalyzed by LarC to that used during the synthesis of nickel-pincer complexes by organometallic chemists. The latter compounds were first synthesized in the 1970s and include a diverse array of structures.[39] Similar to what we propose for LarC, cyclometallation reactions represent a major method for the synthesis of nickel-pincer complexes (Scheme 4A).[35] This reaction has gained interest in the organometallic field since it gives insight into the metal-mediated activation of unreactive C–H bonds.[35b] On the other hand, organometallic chemists have developed alternative synthetic approaches to make nickel-pincer complexes, including oxidative addition using bis(cyclooctadiene)nickel(0) and transmetalation such as when lithium is replaced by nickel (Scheme 4B and 4 C).[40]

Pincer complexes closely resembling the NPN cofactor, with nickel coordinated by the *para*-carbon atom of a pyridinium ring along with two sulfur atoms, were synthesized using two of these nickel insertion approaches. The pyridinium 3,5-bisthioamide nickel complex (Scheme 5A) was generated by oxidative addition using bis(cyclooctadiene)nickel(0).[41] The more recently reported pyridinium 3,5-bisthioacid nickel complexes (Scheme 5B), which better structurally resembles the



Scheme 4. Three routes used for synthesis of metal-pincer complexes. E_1 and E_2 are neutral two-electron donating groups, Y most frequently represents carbon position 1 of a 2,6-disubstituted phenyl ring, and X is often Cl, Br, or I. (A) Cyclometallation using a Ni^{2+} donor. (B) Oxidative addition using a reduced metal species such as the Ni^0 coordinated by two molecules of 1,5-cyclooctadiene. (C) Transmetalation involving initial lithiation followed by replacement of lithium by nickel.



Scheme 5. Structures of two synthetic nickel-pincer complexes resembling the NPN cofactor.

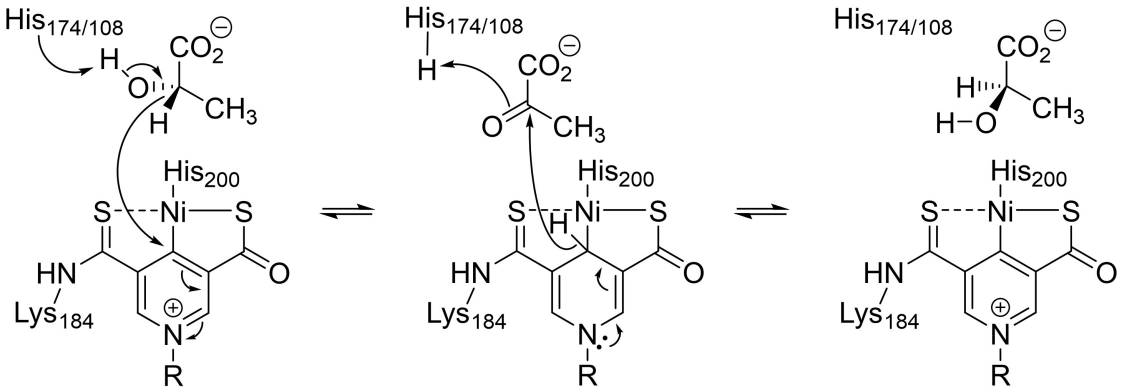
NPN cofactor by replacing the thioamides with thiocarboxylates, were generated by a cyclometallation reaction using Ni $(OAc)_2$.[42] These complexes are useful for providing insight into the mechanism of LarA, which will be discussed in the next section.

3.2. Mechanism of the NPN cofactor in lactate racemization

The unique structure of the NPN cofactor raises the question of how it functions in lactate racemase catalysis. Efforts to investigate the role of the NPN cofactor include reactivity analyses for synthetic model complexes, computational studies, and direct characterization of the enzyme. The following paragraphs discuss each of these topics.

To gain insight into the function of the NPN cofactor of LarA, the reactivities of synthetic analogs were studied. The first-generation NPN analogue (Scheme 5A), containing the 3,5-bisdiethylthioamide modification of an N-methylpyridinium ring, is capable of irreversibly dehydrogenating primary and secondary alcohols in the presence of a strong base (DBU), but is devoid of racemase activity.[41] The second-generation NPN mimic (Scheme 5B), containing two thioacids instead of thioamides, successfully racemizes lactic acid when combined with two equivalents of DBU.[42] Whereas LarA from *L. plantarum* exhibits a k_{cat} of $4{,}745 \pm 544\ s^{-1}$,[29] lactate racemization by the NPN mimic is very slow with a turnover number of 3 after 36 hours.[42] The anionic sulfur ligands in the second-generation analog likely result in a more stable pincer complex compared to the neutral sulfur atoms of the thioamides in the first-generation NPN mimic. The ability of an NPN analog to perform lactate racemization is quite astounding considering the lack of protein residues to orient the substrate and stabilize the transition states.

DFT calculations accompanying the above reports on the synthesis of nickel-pincer complexes[41–42] along with separate computational studies on 63-atom, 139-atom, 200-atom, or other models of the NPN cofactor[43] are consistent with a proton-coupled hydride transfer (PCHT) mechanism for lactate racemization. The PCHT mechanism envisions abstraction of the substrate hydroxyl proton by a general base concomitant with hydride transfer from the α-carbon of lactate to the cofactor, with the hydride returning to either face of the pyruvate intermediate (Scheme 6). This mechanism was proposed when the NPN cofactor was discovered,[16] and that report noted two histidine residues flanking the active site; each of these residues may function as the general base depending on the starting enantiomer of lactate. Calculations using the B3LYP or similar

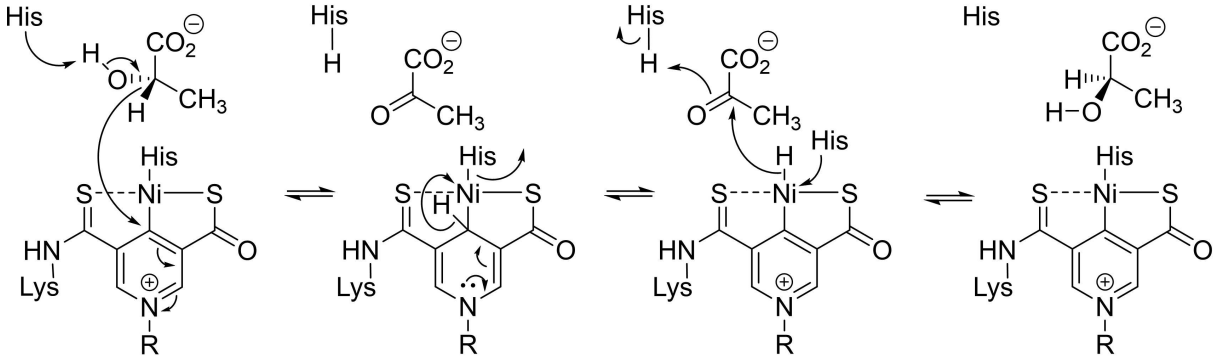


Scheme 6. Postulated proton-coupled hydride transfer (PCHT) mechanism of lactate racemase.

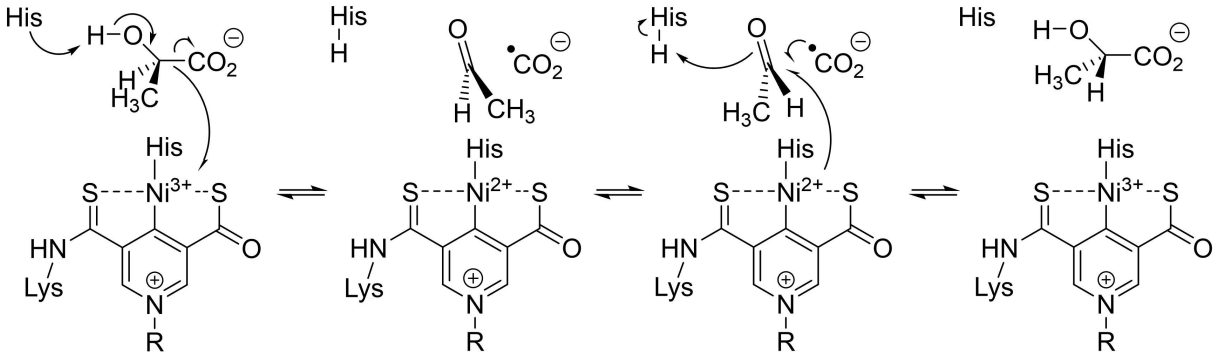
level of theory for the synthetic complexes and models predict the transition state energy for lactate racemization ranges from 12 to 30 kcal/mol. For example, computations involving dehydrogenation of benzyl alcohol by the model compound shown in Scheme 5A are consistent with DBU-catalyzed deprotonation of the alcohol simultaneous with hydride transfer to C2 or C4 of the pyridinium ring with a transition energy of 26 or 26.5 kcal/mol.[41] Similar results were obtained for DFT computations involving the functionally active nickel-pincer complex of Scheme 5B; i.e. base-catalyzed deprotonation of the hydroxyl group combined with hydride transfer to C4 of the pyridinium ring yielded a transition energy of 29.8 kcal/mol.[42] Similar analyses of lactate racemase models suggest the cofactor stabilizes the transition state and predicts lower activation barriers for transfer of hydride to C4, while destabilizing the calculated pyruvate/NPN-hydride intermediate relative to the reactants.[43]

Because nickel-hydride pincer complexes are also well known,[44] it is important to consider whether PCHT could provide an intermediate with hydride coordinating the metal. Calculations for the complexes in Scheme 5 ruled out a pathway involving a nickel-alkoxide intermediate leading to a nickel-hydride species because the transition state barrier is inaccessible (>40 kcal/mol).[41–42] Similarly, two of the model studies suggested a nickel-hydride intermediate is unlikely.[43a,b] We note, however, that the metal remained coordinated to the histidine ligand in those calculations. By assuming the histidine residue can dissociate from the metal during the reaction, calculations revealed that hydride transfer to the nickel center is only 8.4 kcal/mol higher in energy than hydride transfer to C4.[45] A variation of the PCHT mechanism was proposed in which the cofactor can accept a hydride at either C4 or nickel, depending on the isomer of lactate provided, and exchange between these sites within the cofactor facilitates the racemization reaction (Scheme 7). This mechanism provides a rationale for including the nickel atom in the NPN cofactor of lactate racemase. It may be possible to test this hypothesis in LarA by using ^{1}H-NMR to detect the presence of a nickel-hydride, associated with a resonance upfield of tetramethylsilane.[44]

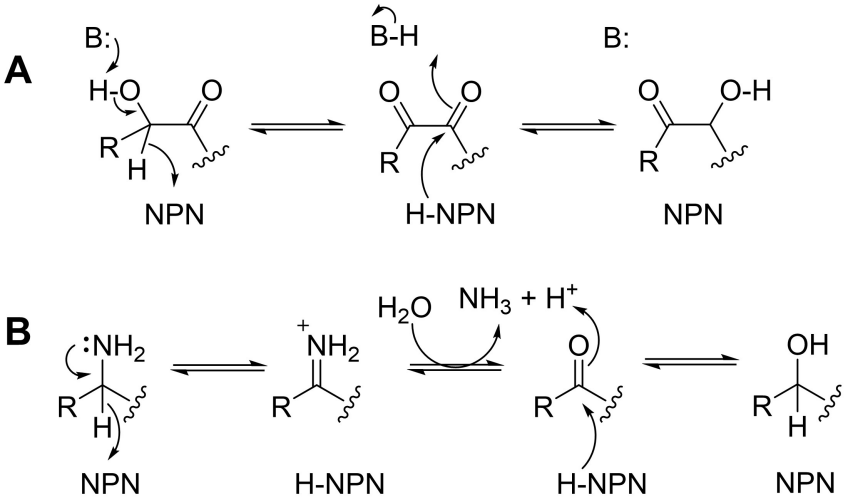
A distinct mechanism for LarA, proton-coupled electron transfer (PCET), was proposed on the basis of quantum mechanics/molecular mechanics calculations.[46] This mechanism also proposes abstraction of the hydroxyl proton of lactate by a general base; however, it suggests this step occurs in concert with the homolytic cleavage of its C1–C2 bond and a 1-electron reduction of Ni(III) to Ni(II), yielding acetaldehyde and a carboxylate radical as intermediates (Scheme 8). Rotation about the carbonyl bond of acetaldehyde followed by Ni(II) oxidation and reconstitution of the C1–C2 bond permits either lactate isomer to form. The calculated energy barrier of hydroxyl group deprotonation and C1–C2 cleavage is 3.1 kcal/mol, whereas



Scheme 7. Alternative hypothesis for a PCHT mechanism in which the hydride can exist at two sites on the NPN cofactor and providing a rationale for the complexity of the NPN structure.



Scheme 8. Hypothetical proton-coupled electron transfer (PCET) mechanism for LarA.



Scheme 9. Hypothetical roles for the NPN cofactor in reactions other than racemization or epimerization. (A) Isomerization. (B) Deamination.

that for reconstituting the C1–C2 bond and reforming lactate is 15.5 kcal/mol.

Experimental studies provided evidence related to the PCHT and PCET mechanisms.[45] The key points distinguishing these proposals are the nickel redox state, the identity of the intermediate, and the substrate bond that is broken/reformed during the reaction. Whereas the PCHT mechanism maintains Ni in the +2 state, PCET stipulates that the resting Ni is +3 (doublet state), and reduced to +2 (singlet state) during catalysis. Electron paramagnetic resonance (EPR) spectroscopy was used to distinguish between these possibilities; no EPR signal is expected for Ni(II), whereas Ni(III) typically exhibits an EPR signal in the range of $g = 2.4–2.0$. Continuous-wave X-band EPR experiments conducted on purified LarA (75 μM), with and without substrate, produced no signal in the 150–450 mT region scanned, thus favoring the PCHT mechanism. Further evidence supporting PCHT over PCET was obtained through efforts to detect the predicted intermediate of the reaction: pyruvate or acetaldehyde, respectively. Pyruvate was detected by coupling the peroxide production by pyruvate oxidase with the horseradish peroxidase-catalyzed oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) that was detected using fluorescence spectroscopy. In addition, pyruvate was shown to form by LC–MS in quenched reactions of purified LarA and pyruvate-free lactate after derivatization with 2,4-dinitrophenylhydrazine. Notably, the amount of pyruvate detected was 5–30%, by mole, of the enzyme. To distinguish whether the C–H or C–C bond is broken, kinetic isotope effect (KIE) studies were carried out using lactic acid with deuterium incorporated at the C2 position. This experiment was performed originally using cell-free lysates from *Clostridium butylicum*, where lactate racemization was associated with KIEs of 2.16 and 2.14 for the D- and L-isomers, respectively.[47] More recent results compared the reaction rates of purified LarA from *L. plantarum* using 2-α-^{2}H–L-lactate versus non-labeled L-lactate, and resulted in a k_H/k_D of 3.11 ± 0.17.[45] This value is within the range (KIE of 3–5) of a hydride transfer in NAD^+ enzymes and provides further evidence for the PCHT mechanism, which predicts C–H cleavage, over the PCET mechanism, which predicts no C–H cleavage.

4. Concluding remarks

Enzymes containing metal-PQQ cofactors are currently limited to those using methanol, ethanol, glycerol, and certain aldehydes as substrates; however, the reaction mechanism shown in Scheme 2 could theoretically apply to a much wider range of primary alcohols or aldehydes along with secondary alcohols. It is plausible that still-to-be-discovered enzymes utilize additional substrates, and one can imagine engineering currently known enzymes to accept a broader range of substrates. A disadvantage of using metal-PQQ-dependent dehydrogenases for biotechnological applications is their requirement for a membrane-bound electron acceptor. Nevertheless, we can expect this group of enzymes containing metal-pincer complexes to be exploited for other desired catalytic purposes.

Characterization of *L. plantarum* lactate racemase with its distinct NPN cofactor leads to questions concerning the prevalence and distribution of the associated genes in Nature. *In silico* analysis of 1,087 bacterial and archaeal genomes found homologues of *larA*, *larB*, *larC*, *larD* (encoding an aquaporin or lactate permease specific to lactic acid), and *larE* in 111, 260, 263, 9, and 259 species, respectively.[29] Intriguingly, many species contain multiple (up to eight) copies of *larA*-like genes, suggesting possible alternative functions for some of the gene products. Furthermore, the finding that many genomes (153 of 1,087 species) contain *larB*, *larC*, and *larE* homologs, but lack *larA* homologs, suggest the use of NPN by non-LarA proteins. It is plausible that related enzymes utilize the NPN cofactor to invert the stereochemistry in other substances containing single

asymmetric carbon atoms (i.e. racemizations) or in molecules containing multiple centers of symmetry (i.e. epimerizations). For instance, a LarA homolog from *Thermotoga maritima* is thought to catalyze the epimerization of D-mannonate to D-gluconate.[48] Other theoretical roles for the NPN cofactor include facilitation of isomerase or deaminase reactions as illustrated in Scheme 9. Further investigations are necessary to establish the breadth of reactions catalyzed by enzymes using NPN as a cofactor. Extending beyond the potential naturally occurring examples, one can imagine the engineering of LarA homologues to provide new routes for synthesis of desired rare sugar isomers.[49]

The demonstration of metal-PQQ cofactors in selected dehydrogenases and the recent discovery of the NPN cofactor in lactate racemase raise the possibility that other types of metal-pincer complexes also exist in biology. The vast knowledge base provided by synthetic inorganic chemists showcase the range of reactions potentially catalyzed by such novel enzymes. We look forward to witnessing further advances in this field.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: metalloenzyme · cofactor · nickel · biosynthesis · biocatalysis

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