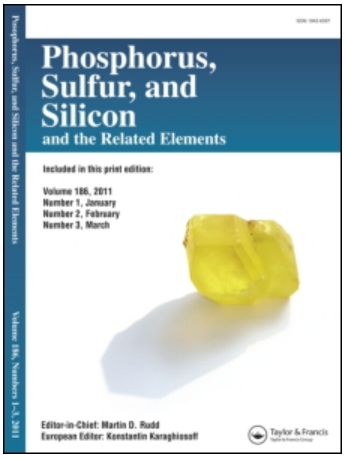

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Generation of the [NiFe] Hydrogenase Active Site (Sulfur's the One!)

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Chemical and biochemical methods were used to unravel the unprecedented pathway by which the CN ligands of iron in [NiFe] hydrogenase are introduced. Carbamoyl phosphate is the one carbon precursor of these ligands, and reactions involving a protein cysteinyl sulfur are key for processing this precursor into CN ligands.

Keywords Carbamoyl phosphate; [NiFe] hydrogenase biosynthesis; thiocarbamate, thiocyanate

INTRODUCTION

Hydrogenases[1] are enzymes widely found in bacteria and archae that catalyze the reduction of protons to dihydrogen and the reverse process

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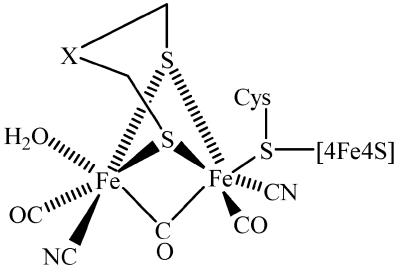
shown in Equation 1.

$$H_2 \rightleftharpoons 2H^+ + 2e^- \quad (1)$$

There has been much recent interest in these enzymes for several reasons. Their active sites are unusual, and the assembly of these active sites is complicated. The mechanism[2] by which these enzymes effect catalysis is important not only for its own sake but for designing simpler, inexpensive, efficient, and robust catalysts for this reaction. There is much interest in developing an energy economy based on hydrogen. Hydrogen is promising as a fuel because it is a renewable energy source and nonpolluting. Catalysis of the production of dihydrogen by the reduction of water as well as the oxidation of dihydrogen by oxygen to produce water and energy is crucial to realizing a hydrogen energy economy. Biocatalysis of hydrogen production and biofuel cells may also play a prominent role in meeting this challenge.[3]

The crystal structures of a number of hydrogenases have been determined. Hydrogenases typically feature a bimetallic active site although "metal free" hydrogenases from archae have been reported. However, recent studies suggest that there is an essential iron even in these hydrogenases.[4] The binuclear hydrogenases form two classes based on the metals occurring at their active sites. The first class is the iron-only hydrogenase, Fe only H_2ase, in which two iron atoms are in the active site shown in **1**.[5] It is currently suggested that X = NH in the enzymes.[6,7] The unusual diiron system bridged by a 2-aza-1,3-propanedithiolate moiety can easily be assembled in model systems. Thus dithiol complex **3**[8] reacts with ammonia and paraformaldehyde to efficiently produce **4** in a process reminiscent of the Mannich reaction.[9,10] The second class of hydrogenases is the nickel iron hydrogenase [NiFe] H_2ase in which an iron and nickel atom are in the active site shown in **2**.[11] In this enzyme, X = OH for its oxidized form and X = H for the reduced form. In another member of this class, [NiFeSe]



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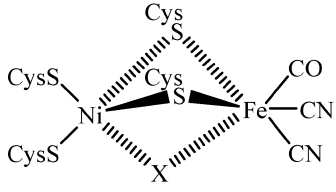

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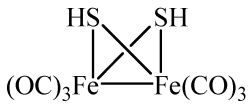
SCHEME 1



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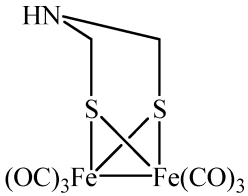
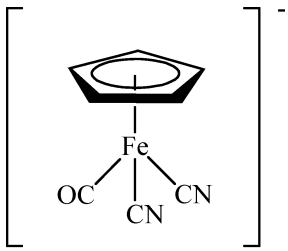


4

SCHEME 2

H_2ase, selenocysteine replaces one of the terminal cysteine residues ligated to nickel.[12]

An unusual feature of the active sites **1** and **2** is that the metals bear CO and $^-$CN ligands. Thus these are organometallic enzymes. This constitutes a second class of biomolecules with carbon-transition metal bonds in addition to cobalamins (B-12). Confirmation of the presence of these unusual ligands at the active site was obtained by Fourier Transform IR spectroscopic analysis.[13] The CO stretching frequency of metal-CO complexes as well as CN stretching frequencies occur in a characteristic region in the infrared where proteins typically do not absorb. The observed stretching frequencies of 1945, 2083, and 2093 cm^{-1} provide excellent evidence for the CO and two CN ligands (the two CN ligands give rise to symmetric and antisymmetric absorptions). This is illustrated by comparison with model compound **5**[14] in which υ_{CO} = 1949 cm^{-1} and υ_{CN} = 2088 and 2094 cm^{-1}. Further evidence for the assignment in the enzyme was obtained by observation of the expected isotope shifts in the ^{15}N and ^{13}C labeled compounds.



5

SCHEME 3

This paper is concerned with the biochemical origins of the CO and CN^- ligands attached to the iron atom of the [NiFe] H_2ase active site. Although such ligands are commonplace in organometallic chemistry, this

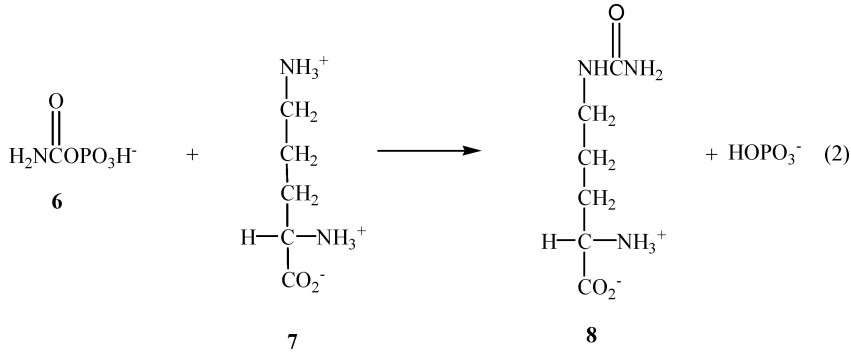
is their first reported occurrence in nature (although cyanocobalamin, Vitamin B-12, features a Co—CN bond, the biological coenzyme has a labile Co—C(5′) adenosyl bond). In organometallic chemistry CO and CN^- ligands can be introduced by treatment of suitable metal species with carbon monoxide or cyanide salts. However both carbon monoxide and cyanide salts are highly toxic. How are these ligands introduced in vivo? Before addressing this issue some salient features about [NiFe] H_2ase and its biosynthesis will be briefly reviewed.[15]

Because the X-ray crystal structure of the [NiFe] H_2ase from *Desulfovibrio gigas* has been reported,[16] its structure will be used to illustrate the essential features of the biosynthesis of these enzymes, even though other enzymes in this class differ in the size of the subunits and FeS cluster content. The *D. gigas* [NiFe] H_2ase consists of a large subunit of 60 kDa and a small subunit of 28 kDa. The active site **2** is attached to the large subunit by the cysteine residues shown in **2** and is buried in the protein. While the protein protects the active site, it raises the question of how the active site is assembled in the midst of the protein. The smaller subunit contains one [3Fe4S] and two [4Fe4S] clusters which provide a conduit for electrons from the surface to the active site. Seven genes producing HypA-F proteins and a specific endopeptidase are required for formation and insertion of the active site. The protein on which the active site is assembled initially has 15 more amino acid residues at its C-terminus than the fully active hydrogenase. In addition the conformation of the large subunit in the enzyme and the "preprotein" are different. The iron is chaperoned by HypC and HypD. Its CO and CN^- ligands are inserted with the involvement of HypF and HypE. The iron is then incorporated into the large subunit precursor which is chaperoned by HypC. The nickel is then added in a GTP-hydrolysis-dependent reaction that involves HypA and HypB. HypC then dissociates and the specific endopeptidase mentioned above cleaves the C-terminal 15 residue polypeptide. This cleavage triggers a conformational change that internalizes the active site. This process simply explains how the active site in center of the hydrogenase is assembled. That is, the conformation of the precursor of the largest subunit permits attachment of the metal centers, and after cleavage of C-terminal 15-amino acid residue, the active site is internalized. The large subunit then heterodimerizes with the small subunit, creating the fully assembled hydrogenase.

RESULTS AND DISCUSSION

Because HypF was known to play a role in the introduction of the CO and CN^- ligands, its sequence was analyzed in the hope of obtaining a

lead on its function. This analysis uncovered an acyl phosphatase motif in HypF. To follow this lead, the protein was first purified. Then the ability of this protein to catalyze the hydrolysis of various phosphates, such as phosphoenolpyruvate, creatine phosphate, acetyl phosphate, glycerol 2-phosphate, and carbamoyl phosphate, was evaluated by monitoring phosphate production.[17] The protein showed modest phosphatase activity except for carbamoyl phosphate. HypF proved to be a selective catalyst for hydrolyzing carbamoyl phosphate. Carbamoyl phosphate, **6**, is a well-known biological species involved in the urea cycle and pyrimidine synthesis. For example, ammonia produced from amino acid metabolism is excreted as urea via the urea cycle. In this cycle, carbamoyl phosphate formed from ammonium salts, carbon dioxide, and ATP enters the urea cycle by reacting with ornithine **7** to afford citrulline **8** as shown in Equation 2. Further steps in this cycle ultimately produce arginine, which is hydrolyzed to urea, thereby regenerating ornithine.



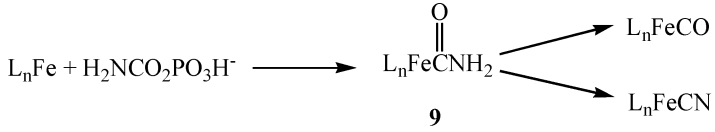
(2)

The selective hydrolysis of carbamoyl phosphate provided the lead that this one-carbon compound might be the biological precursor of the one carbon CO and CN^- ligands in [NiFe] H_2ase. To evaluate this conjecture, the following experiments were done.[18] The specific hydrogenase activity of *E. coli* mutants unable to synthesize carbamoyl phosphate was determined. These mutants were grown in medium in which arginine and uracil was added (because carbamoyl phosphate would not be available for their production). These mutants were devoid of hydrogenase activity. This activity was restored by either reintroducing the transcriptional unit for carbamoyl phosphate synthetase to the mutant or adding L-citrulline to the medium. The addition of L-citrulline provides carbamoyl phosphate by reversal of reaction involved in the urea cycle. Directly observing the [NiFe] hydrogenase isoenzymes after polyacrylamide gel electrophoresis under nondenaturing conditions and staining for hydrogenase activity supported the conclusion that

carbamoyl phosphate is required for the synthesis of active [NiFe] hydrogenase.

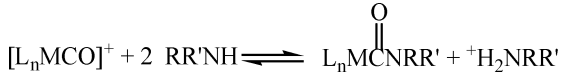
An especially meaningful result was obtained by conducting the experiments outlined above with mutants followed by denaturing (SDS, sodium dodecyl sulfate) polyacrylamide gel electrophoresis and immunostaining with antibodies to the large subunit. In the case of the mutant incapable of carbamoyl phosphate synthesis, a protein was formed that was stained. However, it does not correspond to the processed large subunit but to its precursor. That is, the unavailability of carbamoyl phosphate does not interfere with large subunit synthesis but rather with introduction of the iron ligands. Without the proper ligands on iron, the endopeptidase does not cleave the C-terminal 15-amino acid polypeptide. This is remedied by reintroducing the transcriptional unit for carbamoyl phosphate to the mutant or adding L-citrulline to the medium.

With these biochemical results in hand, the question was raised whether it is chemically reasonable for carbamoyl phosphate to be the source of the CO and CN ligands of iron. A hypothesis based on the well-known but little-studied carboxamido metal species and their chemistry was proposed.[17] Oxidative addition of an iron species to carbamoyl phosphate would generate an iron carboxamido complex **9**. Protonation on nitrogen in **9** renders the amino group a leaving group whose loss generates a carbonyl group as shown in Equation 3.



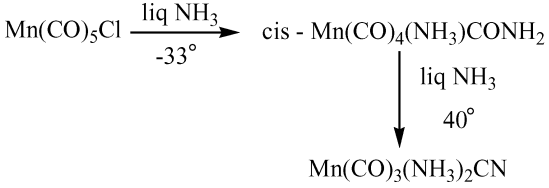
(3)

Both the addition of amines to cationic metal carbonyl complexes and its reverse, shown in Equation 4, are well known.[19] However, dehydration of metal carboxamido species is rarely encountered.



(4)

Chloropentacarbonyl manganese reacts in liquid ammonia at −33°C to form the carboxamido species shown in Equation 5. Upon warming to 40°C, this species dehydrates to afford a nitrile complex.[20]



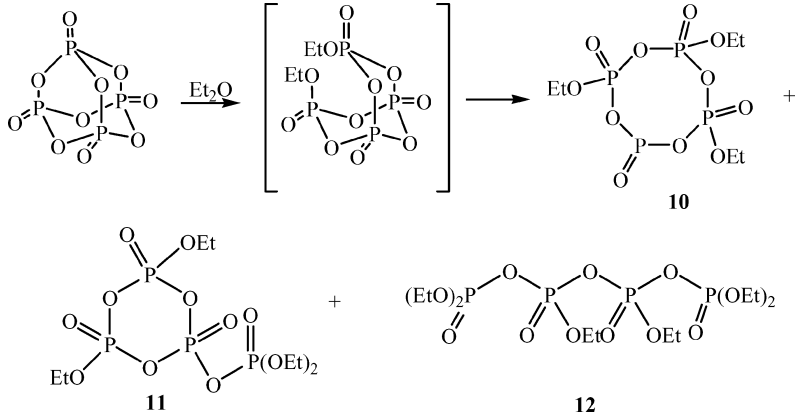
(5)

However, this reaction is not general. Indeed η^5-$CpFe(CO)_2CONH_2$, $FpCONH_2$, formed from $Fp(CO)^+$ in liquid ammonia at $-50^\circ C$, undergoes exclusively β-elimination on warming as shown in Equation 6.[21] Nevertheless, $FpCONH_2$ could be dehydrated to FpCN in 77% yield on treatment with $POCl_3$ and

$$[FpCO]^+ \xrightarrow[-50^\circ]{\text{liq } NH_3} FpCONH_2 \xrightarrow[10^\circ]{\text{liq } NH_3} FpH + NH_4NCO \qquad (6)$$

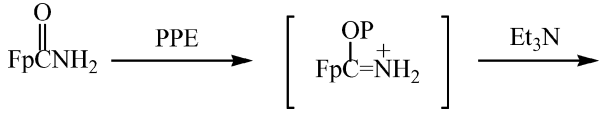
$$Fp = \eta^5\text{-}CpFe(CO)_2$$

Et_3N in dichloromethane. Under physiological conditions ATP would be required to effect this dehydration. Because ATP, being a polyanion, is water soluble but not soluble in organic solvents, a surrogate, which is soluble in organic solvents, is needed. Ethyl polyphosphate was chosen for this purpose. Ethyl polyphosphate (PPE) is formed from P_4O_{10} and diethyl ether as shown in Equation 7 and is a mixture of compounds **10–12** that contain phosphoric acid anhydride bonds like ATP.[22,23]



(7)

It is also known[24] to dehydrate organic nitriles. The reaction of $FpCONH_2$ with ethyl polyphosphate gave an intermediate which afforded FpCN in 57% yield on treatment with Et_3N as shown in Equation 8.



(8)

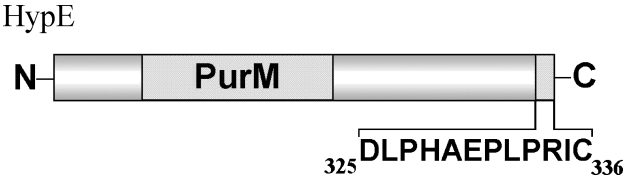
Despite the chemical viability of this pathway, it was discovered that nature adopts an alternative, surprising pathway for processing carbamoyl phosphate into a cyanide ligand. This process and its discovery are presented below.

As already pointed out, HypF is involved with CO and CN ligand formation. Indeed the sequence homology of HypF with acyl phosphatases and its selective phosphatase activity led to the revelation that carbamoyl phosphate might be the one-carbon source of the CO and CN ligands. But HypF has another unusual activity. It catalyzes pyrophosphate exchange with ATP that depends on carbamoyl phosphate. That is, the α,β-phosphoric acid anhydride bond in ATP is cleaved, releasing pyrophosphate, and a new bond is formed with pyrophosphate and the α-phosphate, resulting in ATP. Because carbamoyl phosphate is required for this exchange, it may carbamoylate HypF. Indeed, HypF possesses a sequence motif found in proteins catalyzing O-carbamoylations. To test this hypothesis, HypF was exposed to C-14-labeled carbamoyl phosphate and ATP. However, no radiolabel was covalently incorporated into the protein. A breakthrough in these studies occurred as a result of following up on the report that HypF and HypE interact with each other. Thus exposure of HypF *and* HypE to C-14-labeled carbamoyl phosphate and ATP resulted in covalent incorporation of the radiolabel into HypE. Increasing the concentration of HypE but not HypF increases the incorporation of radiolabel. Omitting HypF results in no incorporation of the radiolabel into HypE. Thus HypF is required for the activation of carbamoyl phosphate. ATP is also essential for this process and its relationship to the HypF catalyzed pyrophosphate exchange was shown in the following way. If ATP is replaced in this reaction (C-14-labeled carbamoyl phosphate, HypF, HypE, and ATP) by its analogue $AMPCH_2PP$, in which the carbon replacing the α,β-anhydride oxygen precludes pyrophosphate cleavage, HypE is not radiolabeled. However, use of $ADPCH_2P$, in which cleavage of the β,γ- but not the α,β-anhydride linkage is precluded, in place of ATP still results in radiolabeling of HypE. These findings show that HypF activates carbamoyl phosphate, which results in the carbamoylation of HypE. A reasonable hypothesis accounting for the facts is shown in Equation 9.

$$\text{HypF} + \text{ATP} + \mathbf{6} \longrightarrow \left[\text{HypF}\bullet\text{AMPO}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{NH}_2\right] + \text{P}_\text{i} + \text{PP}_\text{i} \tag{9}$$

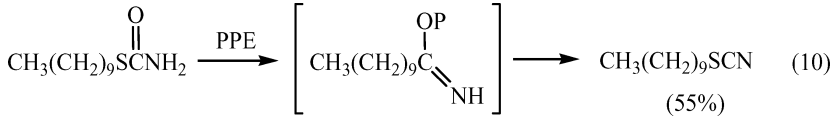
$$\left[\text{HypF}\bullet\text{AMPO}\overset{\overset{\text{O}}{\|}}{\text{C}}\text{NH}_2\right] + \text{HypE} \longrightarrow \text{HypE}(\text{CONH}_2) + \text{HypF} + \text{AMP}$$

Because HypE contains an invariant C-terminal cysteine, it was speculated that the cysteine –SH is carbamoylated. In support of this speculation, it was found that HypE with the cysteine at the C-terminus deleted is not radiolabeled. However, more conclusive evidence was required. Use of modern mass spectroscopic analytic techniques provided proof for this speculation and an unexpected bonus as well. Digestion of HypE with the endopeptidase Asp-N provides a unique fragment that corresponds to the C-terminus of HypE (residues 325–336)[25] and has a $m/z = 1360.7$. A schematic drawing of the HypE sequence is shown in Scheme 5.



SCHEME 4

No similar fragment is obtained from HypF on Asp-N digestion. Consequently this unique fragment allowed its monitoring even in the mixtures of HypE and HypF studied. The reaction of HypF, HypE, carbamoyl phosphate, and ATP followed by Asp-N digestion was analyzed by mass spectrometry. If the speculation that the C-terminal cysteine was carbamoylated was correct, then the 325–336 fragment (m/z 1360.7) should be increased by 43 (44 for $-CONH_2$ and -1 for the H that is replaced by this group). However, instead of $m/z = 1403.7$, a peak with $m/z = 1385.7$ was observed.[18] Thus, there was an increase of 26-1 = 25. This corresponds to addition of CN. The obvious implication is that the expected $HypEcysSCONH_2$ was dehydrated to HypEcysSCN. This proposed dehydration is chemically feasible. Treatment of S-(n-decyl) thiocarbamate with ethyl polyphosphate gave S-(n-decyl) thiocyanate in 55% yield as shown in Equation 10.

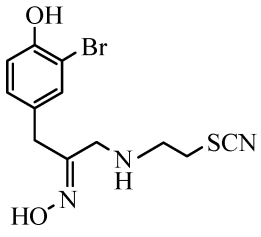


(10)

Furthermore, this dehydration is biochemically feasible because HypE has a sequence similar to the PurM protein that catalyzes an ATP-dependent dehydration. To prove that $HypEcysSCONH_2$ is the precursor of HypEcysSCN, the dehydration must be prevented. In analogy with the PurM protein this dehydration was assumed to be ATP

dependent. Consequently, if ATP is omitted, dehydration would be prevented. But ATP is required for activation of carbamoyl phosphate as outlined previously. How can this conundrum be resolved? The answer was very simple. The activation step requires cleavage of the α,β-phosphoric acid anhydride bond but, presumably, dehydration would require cleavage of the β,γ-phosphoric acid anhydride bond. Indeed, reaction of HypF, HypE, carbamoyl phosphate, and $ADPCH_2P$, in which cleavage can occur between the α,β- but not β,γ-phosphoric moieties, resulted in carbamoylation of HypE without dehydration. That is, after Asp-N protease treatment, mass spectroscopic analysis of the reaction mixture showed a peak with $m/z = 1403.7$. Although these results prove that carbamoylation of HypE occurs on an amino acid residue in the 325–336 C-terminus, it does not establish which amino acid reacts. However, tandem mass spectral (MS-MS) analysis resolved this issue. In this experiment the 325–336 fragment is selected and its fragmentation observed. The 325–336 sequence is DLPHAEPLPRIC. When cleavage occurs the charge can go to amino terminus containing fragment (a,b sequence) or carboxyl terminus containing fragment (y,z sequence). In comparing the unmodified 325–336 fragment, the $CONH_2$ modified fragment, and the CN modified fragment, the modification (increase in m/z) never occurred with the a,b sequence but always occurred with the y,z sequence even on cleavage of the last amino acid. This proved that the modification was attached to the C-terminal amino acid: cysteine. Furthermore, in the $-$CN modified fragment a $[M+H]^+$-HSCN peak was observed, establishing that the –CN and by implication $-CONH_2$ groups were attached to the sulfur of cysteine-336. These results demonstrate that the biological system handles the conversion of carbamoyl phosphate to cyanide in a novel, unanticipated way. The C-terminal cysteine is carbamoylated to give a thiocarbamate. The thiocarbamate is dehydrated to produce a thiocyanate. Thiocyanates are very rare in nature, in contrast to the more commonly occurring isothiocyanates. A few marine natural products have been reported with thiocyanate moieties[26–28] and their structures **13–15** are shown in (Scheme 5). It is noteworthy that the processing of carbamoyl phosphate to a CN moiety occurs while attached to sulfur, thereby avoiding generation of lethal cyanide ion.

Although the detailed biochemical process by which HypEcysSCN transfers CN to Fe of the emerging hydrogenase has not been discovered as yet, models support the chemical feasibility of this process. This process involves a $2e^-$ reduction coupled with the CN transfer from S to Fe to generate HypEcysSH and L_nFeCN. Two mechanistic extremes may be envisioned: (1) $2e^-$ reduction of the –SCN moiety coupled with formally "$^-$CN" transfer to electrophilic Fe, (2) $2e^-$ reduction of Fe to



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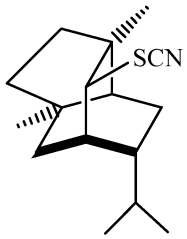

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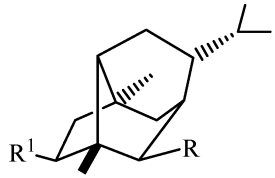

15a, R=SCN, R^1=H
b, R=H, R^1=SCN

SCHEME 5

generate a nucleophilic Fe which displaces CN from the –SCN moiety (formally "$^+$CN" is transferred.) Reaction of PhS^- and PhSCN in the presence of FpBr afforded FpCN in 50% yield as shown in Equation 11.

$$PhS^- + PhSCN + FpBr \longrightarrow \underset{(50\%)}{FpCN} + PhSSPh + Br^- \qquad (11)$$

This demonstrates the chemical feasibility of transferring "$^-$CN" from a thiocyanate to electrophilic Fe. Reaction of Fp^- with PhSCN gave a mixture of products resulting from nucleophilic displacement on S and apparently electron transfer as shown in Equation 12. Consequently, Fp^- was treated with tBuSCN.

$$Fp^- + PhSCN \longrightarrow \underset{(1\%)}{FpSPh} + \underset{(40\%)}{Fp_2} + \underset{(57\%)}{PhSSPh} \qquad (12)$$

It was anticipated that this thiocyanate would be a poorer electron acceptor than PhSCN and that the bulky tBu group would disfavor nucleophilic attack on S. Indeed this reaction resulted in the formation of FpCN in about 20% yield without optimization as shown in Equation 13. This result demonstrates the feasibility of nucleophilic iron displacing "$^+$CN" from a thiocyanate.

$$Fp^- + tBuSCN \longrightarrow \underset{(20\%)}{FpCN} + tBuS^- \qquad (13)$$

In summary we have found that the biochemical origin of the CN ligands attached to the Fe of the active site of [NiFe] hydrogenase is carbamoyl phosphate. In addition, carbamoyl phosphate is processed to the CN ligand of Fe by the steps shown in Equations 14–17. Studies

on the biosynthesis of the CO ligand are continuing.

$$\text{HypF} + \text{H}_2\text{NCO}_2\text{PO}_3\text{H}^- + \text{ATP} \longrightarrow [\text{HypF} \cdot \text{AMPOC}(=\text{O})\text{NH}_2] + \text{P}_i + \text{PP}_i \quad (14)$$

$$\text{HypE} + [\text{HypF} \cdot \text{AMPOC}(=\text{O})\text{NH}_2] \longrightarrow \text{HypEcysSCONH}_2 + \text{HypF} + \text{AMP} \quad (15)$$

$$\text{HypEcysSCONH}_2 + \text{ATP} \longrightarrow \text{HypEcysSCN} + \text{ADP} + \text{P}_i \quad (16)$$

$$\text{HypEcysSCN} + \text{L}_n\text{Fe} \xrightarrow{2e^-} \text{HypEcysSH} + \text{L}_n\text{FeCN} \quad (17)$$

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