A Tale of Two Metals:

Come to Cobalt: Synthesis and Characterization of Novel Cobalt-SNS Complexes for H₂ Fuel Catalysis

and

Degradation of Organophosphonates: Study and Characterization of a Novel Fusion Diiron Oxygenase from *F. multimorphosa*.

Master’s Thesis

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Chapter 1. Come to Cobalt: Synthesis and Characterization of Novel Cobalt-SNS Complexes for H₂ Fuel Catalysis

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Catalyst development for acceptorless dehydrogenation of H₂-dense solids such as ammonia borane (NH₃BH₃) moves toward a future using H₂ fuel. Initial synthesis of a novel family of Co³⁺ catalysts using sulfur-nitrogen-sulfur (SNS) ligands proposes several complexes useful for future catalytic cycles due to the hemi-labile and inner-sphere base character of the SNS ligand. One such complex, proposed to be Co(SNSMe)(dmpe), was successful in complete ammonia borane dehydrogenation, but showed signs of catalyst decomposition. We report here the synthesis of the precursor to this successful catalyst, and show the efficient and clean synthesis of a new paramagnetic Co³⁺(N₂S₂) complex.

Synthesis of a thiol SNHSMe heterocyclic ligand is shown with its subsequent ring-opening deprotonation that yields a lithium-THF dimer, characterized by X-ray crystallography and NMR spectroscopy. Coordination of this anionic ligand to cobalt results in an internal ligand imine coupling to produce a cobalt(III) square planar complex: [Co(SNSMe)₂][(THF)₃Li]₂(µ-Cl)]. This paramagnetic complex is characterized by NMR spectroscopy, X-ray crystallography, Evans’ NMR spectroscopy method, and cyclic voltammetry, and relevant data are discussed herein. This work provides one of the first cobalt-SNS precatalysts for NH₃BH₃ dehydrogenation, and opens the door for new cobalt-SNS complexes as potential precatalysts for reductive processes.
## Contents

Acknowledgements .................................................................................................................. iii

Chapter 1. Come to Cobalt: Synthesis and Characterization of Novel Cobalt-SNS Complexes for H₂ Fuel Catalysis ........................................................................................................ v
  Introduction .......................................................................................................................... 1
  Results and Discussion ....................................................................................................... 9
  Materials and Methods ..................................................................................................... 22
    General ............................................................................................................................ 22
    Synthesis ......................................................................................................................... 22
  Conclusion ......................................................................................................................... 28
  Supplemental Spectra: ...................................................................................................... 29
  References ......................................................................................................................... 32

Chapter 2. Degradation of Organophosphonates: Study and Characterization of a Novel Fusion Diiron Oxygenase from F. multimorphosa. ........................................................................ 34
  Introduction ....................................................................................................................... 35
  Results and Discussion ..................................................................................................... 37
  Experimental ....................................................................................................................... 51
    General ............................................................................................................................ 51
    1. Protein expression and purification ............................................................................. 52
    2. Generation of Fm FeOf variants .................................................................................. 56
    3. Thermal shift assays ................................................................................................. 57
    4. Activity Assays .......................................................................................................... 58
    5. Generation of the different redox forms of Fs FeOf ..................................................... 59
    6. Stop-flow Absorption (SF-Abs) spectroscopy ............................................................ 59
    7. Mössbauer spectroscopy .......................................................................................... 61
  Conclusions ......................................................................................................................... 62
  References ......................................................................................................................... 63
List of Figures

Figure 1. $\text{H}_3\text{NBH}_3$ dehydrogenation yields a mix of various products, including linear polymers and cyclic aminoborane, borazine, and polyborazylene. 2

Figure 2. (a) Goldberg’s $\text{Ir(POCOP)}$ catalyst.\textsuperscript{5} (b) $\text{Rh(POCOP)}$ hydride reported by Esteruelas \textit{et al.}\textsuperscript{11} 3

Figure 3. (a) Cobalt(I) catalyst with reversible coordination of $\text{H}_2$ reported by Peters \textit{et al.}\textsuperscript{19} (b) $\text{Cp and Cp}^*$ half-sandwich cobalt(III) complexes reported by Waterman \textit{et al.}\textsuperscript{15} 4

Figure 4. Cobalt hydrogenation and dehydrogenation catalysts. (a) Hanson’s cobalt(II) hydrogenation catalyst.\textsuperscript{14} (b) Hanson’s cobalt(III) alcohol dehydrogenation catalyst.\textsuperscript{14} 5

Figure 5. Binding of SNS$^\text{Me}$ to generic metal (M), showing the prospective hemi-lability of the 6-membered chelate ring versus the increased strength of the 5-membered planar ring. 6

Figure 6. (a) Ring-opened $\{[\text{SNS}^\text{Me}][\text{Li(THF)}]\}_2$ dimer. (b) Imine-coupled ligand coordinated to cobalt, yielding $\text{[Co(SNS}^\text{Me})_2][(\text{THF})_3\text{Li}]_2(\mu-\text{Cl})]$. 8

Figure 7. $^1\text{H NMR}$ spectrum (400 MHz, CD$_2$Cl$_2$) of ring opened ligand, $\{[\text{SNS}^\text{Me}][\text{Li(THF)}]\}_2\text{THF}$. *denotes CD$_2$Cl$_2$. 12

Figure 8. ORTEP diagram of $\{[\text{SNS}^\text{Me}][\text{Li(THF)}]\}_2\text{THF}$. 13

Figure 9. $^1\text{H NMR}$ spectrum (400 MHz, CD$_3$CN) of $\text{[Co(SNS}^\text{Me})_2][(\text{THF})_3\text{Li}]_2(\mu-\text{Cl})]$: *denotes CD$_3$CN solvent; † denotes trace amounts of residual solvents benzene, diethyl ether, dichloromethane, toluene, and pentane. 16

Figure 10. ORTEP diagrams of $\text{[Co(SNS}^\text{Me})_2][(\text{THF})_3\text{Li}]_2(\mu-\text{Cl})]$. (a) Structure of a single $\text{[Co(SNS}^\text{Me})_2]$ anion. (b) Structure of the asymmetric unit, which includes two $\text{[Co(SNS}^\text{Me})_2][(\text{THF})_3\text{Li}]_2(\mu-\text{Cl})]$. 17
Figure 11. Cyclic voltammogram of [Co(SNS^{Me})_{2}][(THF)_{3}Li]_{2}(\mu-Cl)] dissolved in 0.4M TBA-PF_{6}•THF, scanned from -3.25 V to 0 V at 0.1 V s^{-1}, reported versus Fc/Fc^{+}.

Figure 12. Cyclic voltammogram of [Co(SNS^{Me})_{2}][(THF)_{3}Li]_{2}(\mu-Cl)] dissolved in 0.4M TBA-PF_{6}•THF, scanned from -1.25 V to 0.35 V at 0.1 V s^{-1}, reported versus Fc/Fc^{+}.

Figure 13. Homology model of FeOf protein based on similarity to known structural sequences in the pdb database, SnoK and PhnZ proteins.

Figure 14. Mössbauer spectrum of FeOf protein expressed in M9 minimal media with ^{57}Fe.

Figure 15. Melting curves comparing FeOf, PhnY, and PhnZ proteins showing increased overall stability of FeOf protein compared to its PhnY/Z counterparts.

Figure 16. Selected melting curves for the FeOf protein at different pH’s.

Figure 17. FeOf catalyzed conversion of 2-AEP to phosphate (P_{i}) for 2 hours measured by $^{31}$P $^{1}$H NMR spectra (121 MHz, 20% D_{2}O), at 25°C.

Figure 18. FeOf catalyzed conversion of 2-AEP to phosphate (P_{i}) at different time points measured by $^{31}$P $^{1}$H NMR spectra (121 MHz, 20% D_{2}O), at 25°C.

Figure 19. Structures of different phosphonates used for screening of the physiological substrate for the FeOf fusion protein.

Figure 20. $^{31}$P $^{1}$H NMR spectra (121 MHz, 20% D_{2}O), at 25°C measuring product formation in multiple turnover assays screening for physiological substrates.

Figure 21. Competition activity assay between 3-APP and 2-AEP.

Figure 22. $^{31}$P $^{1}$H NMR spectra (121 MHz, 20% D_{2}O), at 25°C comparing activity of the FeOf protein and the PhnY/Z system using 3-APP as a substrate. (a) PhnY/Z system (b) FeOf protein.

Figure 23. Set up for stopped-flow kinetics experiments.
Figure 24. Stop-flow absorption spectra showing the binding of OH-AEP to the HD-domain of the FeOf protein. (a) Relative absorbance (240-720 nm). (b) Difference spectrum. 48

Figure 25. Stopped-flow kinetic trace of the binding of 500 µM OH-AEP to FeOf protein. 48

Figure 26. Determination of $K_d$ by plotting $K_{obs}$ versus substrate concentration. 49

Figure 26. Stopped-flow absorption spectra single turnover experiment (a) Relative absorbance (240-720 nm). (b) Difference spectrum. 50

Figure 28. Stopped-flow absorption spectrum at 397 nm: single turnover experiment. 50

Figure 29. SDS-PAGE gel of FeOf protein, as isolated. 54

Figure 30. SDS-PAGE gel of FeOf protein purification using S200 column. 55

Figure 31. SDS-PAGE gel of Fe$^{57}$Of protein, as isolated. 61

Schemes

Scheme 1. Straightforward and facile synthesis of $S^{Me}N^H_S$ ligand in high yields.21 6

Scheme 2. Reaction of the $S^{Me}N^H_S$ ligand with iron triflate to generate tri-, di-, and mononuclear iron complexes.21 7

Scheme 3. Catalytic dehydrogenation of $NH_3BH_3$ using proposed (SNS$^{Me}$)Co(dmpe). 8

Scheme 4. Synthesis route to proposed Co$^I$(SNS$^{Me}$) complex using (PPh$_3$)$_3$CoCl as a starting material. 10

Scheme 5. Mechanism showing $S^{Me}N^H_S$ ring opening upon deprotonation. 10

Scheme 6. Reaction of the $S^{Me}N^H_S$ ligand with LiHMDS forms the anionic ($S$S$^{Me}$)S$^{Me}$ lithium THF-adduct bridged by thiolates. 11

Scheme 7. Reaction of 1 with CoCl$_2$ in the presence of excess zinc to form the $N_2S_2$ square planar cobalt(III) complex 2. 14

Scheme 8. Control reactions of CoCl$_2$ with the $S^{Me}N^H_S$ ligand and the anionic ligand 1. 15
Scheme 9. Coordination and imine coupling of 2,3-dihydro-2-phenylbenzothiazole in the presence of cobalt(II).

Scheme 10. PhnY hydroxylates 2-AEP to produce OH-AEP, which is broken down into glycine and phosphate by PhnZ. 3

Tables

Table 1. Average bond angles around the cobalt metal center in complex 2.

Table 2. Average bond lengths for Co-N and Co-S bonds for complex 2.

Table 3. Equivalents of iron per protein (as isolated, purified, and after EDTA chelation)

Table 4. Fm FeOf variants H117A and D335A showing wild type, forward mutation, reverse and reverse complementary sequences.

Supplementary Figures

Figure S1. 11B NMR (96 MHz) spectra showing of ammonia borane dehydrogenation catalysis at room temperature using proposed [SNSMe]CoI(dmpe).

Figure S2. 1H NMR spectrum (400 MHz, C6D6) of SNHSM Me ligand. *denotes NMR solvent.

Figure S3. 1H NMR spectrum (400 MHz, CD2Cl2) of proposed [SNSMe][PPh3Co(I)]. *denotes CD2Cl2; † denotes solvent impurities benzene, toluene, and pentane.

Figure S4. UV-vis spectrum of [Co(SNSMe)2][(THF)3Li]2(µ-Cl)] (1 x 10^-4 M in THF): molar extinction coefficient plotted against wavelength (nm).
Introduction

Our global economy requires multi-faceted solutions to our continued reliance on fossil-based hydrocarbons for energy, chemicals, and other products. As noted in several reports published in recent years by the Department of Energy (DOE)\textsuperscript{a}, this is particularly important in the fuel and transportation industries as worldwide energy demands continue to increase.\textsuperscript{1-3} Development of efficient and carbon neutral (or free) alternative fuels will improve environmental sustainability and increase energy independence in the U.S. and worldwide.\textsuperscript{1-3}

Inorganic catalysis provides some of the most promising routes for finding new renewable and abundant ways to provide fuels and chemical feedstocks. Many of these approaches depend on activation and conversion of small molecules, such as hydrogen gas, water, or carbon dioxide gas.\textsuperscript{1-2} Hydrogen fuel has been suggested as an attractive alternative fuel, as its combustion produces only water instead of carbon dioxide.\textsuperscript{2-3} Furthermore, production of H\textsubscript{2} by water splitting could be a convenient way to store energy, addressing excess grid energy in a future powered by environmental renewable energy sources (e.g. solar, wind, etc.), and providing readily available stored energy for excess demand.\textsuperscript{1-3}

However, a hydrogen fuel economy remains elusive, as pressurized or liquefied hydrogen gas (H\textsubscript{2}) storage poses significant storage and safety risks.\textsuperscript{2-6} Furthermore, compression of H\textsubscript{2} results in significant energy loss, and increases the cost for commercial transfer of hydrogen fuel, thus preventing its economic feasibility.\textsuperscript{2-3} One possible solution to this problem is the use of hydrogen storage molecules that can store and release H\textsubscript{2} upon chemical activation.\textsuperscript{2,4-6} Catalytic

\textsuperscript{a} Reports referenced were produced by the Basic Energy Sciences Advisory Committee for the United States Department of Energy.
cycles for hydrogen dense solids will enable H\(_2\) use for both energy storage and as a fuel; however, an effective hydrogen storage molecule requires a high percentage of hydrogen by weight to be useful (BESAC suggested >10% in their 2003 report).\(^2,6-7\)

Ammonia borane (H\(_3\)NBH\(_3\)) has been identified as a target molecule for hydrogen storage: a stable solid at room temperature, it is an efficient hydrogen storage molecule, with 19.6% hydrogen by weight.\(^2,4-8\) H\(_3\)NBH\(_3\) has a melting point of >104°C; this increase in stability compared relatively to ethane (gaseous at room temperature, with a melting point of -181°C) is suggested to be due to the dihydrogen bonding in H\(_3\)NBH\(_3\).\(^6-7,9\) Dihydrogen bonding is described by Crabtree \textit{et al}. as hydrogen bonding between two respectively hydridic and protic hydrogen atoms, with an interatomic distance < 2.2 Å.\(^9,10\) This added stability via dihydrogen bonding allows H\(_3\)NBH\(_3\) to be useful as a solid storage molecule for hydrogen, yet under catalytic dehydrogenation conditions, it has been shown to release >18% H\(_2\) by weight.\(^4,6\) Moreover, metal-catalyzed dehydrogenation allows for control of the rate of H\(_2\) release.\(^6\)

\[ \text{H}_3\text{B}-\text{NH}_3 \stackrel{[\text{M}]}{\longrightarrow} \text{H}_2 + \text{products} \]

*Figure 1.* H\(_3\)NBH\(_3\) dehydrogenation yields a mix of various products, including linear polymers and cyclic aminoborane, borazine, and polyborazylene.\(^7\)

Dehydrogenation of ammonia borane can produce aminoborane (H\(_2\)NBH\(_2\)) or iminoborane (HNBH), but usually results in a mix of the two in linear, branched and cross-linked polymer forms (Figure 1).\(^7-8\) Production of borazine and polyborazine products yield the greatest amount of H\(_2\) per equivalent of H\(_3\)NBH\(_3\).\(^7-8\) It is important to note that one of the principal
challenges in the use of H₃NBH₃ as a hydrogen fuel source is regeneration of ammonia borane.⁶-⁷ Simple rehydrogenation from dehydrogenation products to H₃NBH₃ requires large energy input. Thus far, few feasible systems for regenerating H₃NBH₃ are reported.⁷

Catalytic dehydrogenation of ammonia borane has so far been mostly dominated by late transition precious metals, such as rhodium and iridium.⁵-⁷ Several Rh and Ir complexes in group 9 have been reported as particularly effective in dehydrogenation catalysis of H₃NBH₃.⁵-⁶ For example, Goldberg et al. reported the use of a tridentate phosphite-carbon-phosphite (POCOP) ligands in the synthesis of (POCOP)Ir(H)₂. This complex (Figure 2a) served as a highly active catalyst in H₃NBH₃ dehydrogenation, releasing stoichiometric H₂ gas within 4 minutes at 1.0 mol % catalyst loading.⁵ Esteruelas et al. reported a POCOP-rhodium hydride complex (Figure 2b) effective for H₃NBH₃ dehydrogenation¹¹, and similar POCOP-rhodium complexes have been reported for dehydrogenation of amine-boranes (Weller et al. 2014).¹²

![Figure 2](image-url)

**Figure 2.** (a) Goldberg’s Ir(POCOP) catalyst.⁵ (b) Rh(POCOP) hydride reported by Esteruelas et al.¹¹

1ˢᵗ row transition metals coordinating poly-dentate ligands are beginning to break ground in H₃NBH₃ dehydrogenation. First row transition metals, e.g., Fe, Co, and Ni, are inexpensive, abundant and less toxic than precious metals and are therefore desirable as catalysts.¹³-¹⁵ Rossin et al. suggest that stabilizing, poly-dentate donor ligands may be effective in synthesizing 1ˢᵗ row transition metal catalysts for NH₃BH₃ dehydrogenation.⁶ Baker et al. reported in 2007 a highly active nickel N-heterocyclic carbene (NHC) complex capable of producing over 18% wt. H₂ gas from H₃NBH₃ dehydrogenation in 4 hours.⁴ Iron complexes coordinating tridentate phosphorous-
carbon-phosphorous (PCP) ligands have been known to dehydrogenate H$_3$NBH$_3$ effectively; yet, their cobalt (PCP) counterparts do not.$^6,^6$ Several examples of heterogeneous cobalt catalysts, mainly using cobalt nanoparticles, are reported in the literature to generate hydrogen gas in minutes from efficient hydrolysis of aqueous H$_3$NBH$_3$.$^{17-18}$ However, few homogenous cobalt catalysts for NH$_3$BH$_3$ dehydrogenation have been reported, and this is especially odd given the success of 2$^{nd}$ and 3$^{rd}$ row group 9 late transition metals, as discussed above (e.g., iridium, rhodium).

To date, only two examples of cobalt-based homogenous catalysts for ammonia borane dehydrogenation have been reported. Lin and Peters reported a cobalt(I) bis(phosphino)boryl complex (CoPBP) capable of amine-borane dehydrogenation via reversible coordination of H$_2$ (Figure 3a).$^{19}$ More recently, Waterman et al. reported two half sandwich cobalt(III) complexes, Cp*Co(CO)$_2$I$_2$ and CpCo(CO)$_2$I$_2$, both air-stable complexes that were capable of effecting complete dehydrogenation of H$_3$NBH$_3$ in under 2 hours at 1% catalyst loading (Figure 3b).$^{15}$

![Figure 3](image_url)

**Figure 3.** (a) Cobalt(I) catalyst with reversible coordination of H$_2$ reported by Peters et al.$^{19}$ (b) Cp and Cp* half-sandwich cobalt(III) complexes reported by Waterman et al.$^{15}$

Cobalt-based dehydrogenation and hydrogenation catalysts supported by poly-dentate ligands have tremendous potential in areas beyond H$_3$NBH$_3$ dehydrogenation. Both Peters’ and Waterman’s catalysts were effective in olefin hydrogenation and transfer hydrogenation.$^{15,19}$ However, perhaps more striking are the versatile catalysts reported by Hanson et al.$^{14}$ Figure 4a shows a Co$^{II}$ complex coordinating a tridentate pincer phosphorous-nitrogen-phosphorous (PNP) ligand reported by Hanson et al., capable of olefin and ketone hydrogenation.$^{14}$ The same
catalyst also functioned as a precursor to a Co$^{\text{III}}$ catalyst capable of alcohol dehydrogenation, aided by ligand-metal cooperativity (Figure 4b). With the wealth of cobalt catalysts for hydrogenation and dehydrogenation catalysis, development of a cobalt catalyst capable of H$_3$NBH$_3$ dehydrogenation would fill a large gap in the repertoire of base metal catalysts for H$_2$ fuel catalysis.

**Figure 4.** Cobalt hydrogenation and dehydrogenation catalysts. (a) Hanson’s cobalt(II) hydrogenation catalyst. (b) Hanson’s cobalt(III) alcohol dehydrogenation catalyst.

In designing new polydentate ligand systems for new catalysts targeting energy conversion and storage, one quickly runs into comparisons and analogies to photosynthesis and biological systems. For example, the active site in iron and cobalt nitrile hydratases consist of a metal center supported by three sulfur atoms from cysteine residues, and two nitrogen atoms from carboxamide groups. Based in part on sulfur and nitrogen ligand complexes in enzymes, Baker *et al.* designed and prepared a new tridentate sulfur-nitrogen-sulfur ligand, 2-(2-methylthiophenyl) benzothiazolidine (S$^{\text{Me}}$N$^{\text{H}}$S, Figure 5).

Outside of biology, use of SNS ligands for hydrogenation catalysis has precedent: Page *et al.* reported the use of a Ru(SNS$^{\text{Bu}}$) complex coordinating 2,6-bis(tert-butylthiomethyl)pyridine for transfer hydrogenation of acetophenone.
Figure 5. Binding of SNS\textsuperscript{Me} to generic metal (M), showing the prospective hemi-lability of the 6-membered chelate ring versus the increased strength of the 5-membered planar ring.

The S\textsuperscript{Me}N\textsuperscript{H}S ligand is synthesized easily in excellent yields from inexpensive starting materials (Scheme 1), and combines ‘hard’ nitrogen and ‘soft’ sulfur donors.\textsuperscript{21} Coordinated to a metal center, the anionic thiolate ligand allows the potential for ligand cooperativity, perhaps as an inner-sphere base in hydrogenation/dehydrogenation catalysis. Additionally, the ligand is asymmetric, forming a stronger five membered chelating ring on one side and a weaker six membered ring on the other (Figure 5): this, in addition to the weaker donor properties of the thioether arm, might allow for the ligand to be hemilabile.\textsuperscript{21} This hemilability could help facilitate 2e\textsuperscript{−} transformations in dehydrogenation and hydrogenation catalytic cycles through dissociation and coordination of the ligand.\textsuperscript{22}

Scheme 1. Straightforward and facile synthesis of S\textsuperscript{Me}N\textsuperscript{H}S ligand reported by Baker \textit{et al.} in high yields.\textsuperscript{21}

Uttam Das in the Baker group at the University of Ottawa has fully characterized a series of novel mono-, di-, and tri-nuclear complexes bridged by or coordinating the new SNS\textsuperscript{Me} ligand (Scheme 2).\textsuperscript{21} These examples show that the SNS\textsuperscript{Me} ligand is successful in coordinating as a tridentate ligand\textsuperscript{21}, which could help stabilize the catalyst under dehydrogenation conditions.\textsuperscript{6}
Scheme 2. Reaction of the $S_{MeN}^{Me}$ ligand with iron trflate in the presence of base afforded a tri-nuclear iron(II) complex bridged by thiolate sulfurs. Exposing this complex to ligand donors in the presence of another equivalent of base yielded mono and di-nuclear iron(II) complexes. This work was carried out by graduate student Uttam Das at the University of Ottawa.\textsuperscript{21}

Given the prospective versatility of the SNS ligands designed by Baker \textit{et al.}, development of novel cobalt-SNS complexes aimed at hydrogenation catalysis could target a variety of substrates. Preliminary research carried out at the University of Ottawa investigated whether cobalt could coordinate various SNS ligands. This work resulted in the successful synthesis of a series of proposed Co-SNS complexes supported by chelating ancillary phosphine ligands (e.g. dimethylphosphinoethane, dicyclopophosphinoethane, etc). Herein, we consider primarily the coordination of the $S_{MeN}^{Me}$ ligand described above to cobalt. Successful coordination of the $S_{MeN}^{Me}$ ligand to cobalt, in the presence of base, afforded an unknown paramagnetic species. In the presence of the chelating ligand dimethylphosphinoethane (dmpe), this proposed complex (Scheme 3, below) showed preliminary activity in NH$_3$BH$_3$ dehydrogenation at room temperature over 8 h, producing borazine and cross-linked borazine products (see supplementary data S1). Unfortunately, this active catalyst also decomposed during dehydrogenation, producing dmpe-borane byproducts as well (Figure S1).
Scheme 3. Catalytic dehydrogenation of ammonia borane using (SNSMe)Co(dmpe). Structure proposed for cobalt catalyst based on NMR characterization.

Given the decomposition of the proposed (SNSMe)Co(dmpe) complex, and the successful iron complexes coordinating the anionic SNSMe ligand, it is of interest to identify and characterize the intermediate cobalt complex coordinating the anionic SNSMe ligand without the phosphine. Therefore, herein the coordination of the deprotonated SNSMe ligand to cobalt is described, with reported synthesis and characterization of the resulting CoIII(N2S2) complex by X-ray crystallography, NMR and UV-vis spectroscopy, and cyclic voltammetry. It was found that upon coordination of the SMeNHSS ligand to cobalt in the presence of base, the ligand first undergoes ring-opening and deprotonation, and then dimerizes by coupling at the imine carbon position to form a tetradentate ligand-bound CoIII(N2S2) complex (Figure 6b). Characterization of the ring-opened ligand (Figure 6a) as a Li(THF)-bound dimer is also reported, with X-ray crystallographic structural and NMR spectroscopic data.

**Figure 6.** (a) Ring-opened \([\text{SNSMe}][\text{Li(THF)}]_2\) dimer. (b) Imine-coupled ligand coordinated to cobalt, yielding \([\text{Co(SNSMe)}_2](\text{THF})_2\text{Li}_2(\mu-\text{Cl})\). Counter-ion structure omitted here for clarity.
To our knowledge, this work reports the first example of a Co-SNS complex for NH$_3$BH$_3$ dehydrogenation, and the third example of a homogenous cobalt catalyst for NH$_3$BH$_3$ dehydrogenation. Furthermore, the structure for the $\{[\text{SNS}^{\text{Me}}][\text{Li(THF)}]\}_2$ dimer confirms that the S$^{\text{Me}}$N$^\text{H}$S ligand reported by Baker et al. must undergo ring-opening before coordination to metal centers. Characterization of the precursor to the proposed Co(SNS$^{\text{Me}}$)(dmpe) complex reveals an imine coupled ligand dimer coordinating to a cobalt(III) center in a square planar geometry. This isolated and characterized complex opens the door to a series of novel Co-SNS complexes that hold potential for dehydrogenation and hydrogenation catalysis. The versatility in catalysis effected by cobalt complexes discussed in the literature with markedly different ligand environments and oxidation states suggests that the Co-SNS complex described here could have potential far beyond simple NH$_3$BH$_3$ dehydrogenation. Indeed, the isolated complex itself may be an active catalyst, and future studies will investigate its catalytic scope and potential.

**Results and Discussion**

As shown in Scheme 1, the S$^{\text{Me}}$N$^\text{H}$S ligand was prepared in high yield and purity from a simple condensation reaction of thiobenzaldehyde and 2-aminothiophenol in ‘super-dry’ ethanol, following the procedure reported by Baker et al.$^{21}$ The NMR spectrum of the ligand in C$_6$D$_6$ matched the spectrum reported by Baker et al (Figure S2)$^{21}$.

Attempts at synthesizing the Co$^1$(SNS$^{\text{Me}}$) precursor to the (SNS$^{\text{Me}}$)Co$^1$(dmpe) complex shown in Scheme 1 used tris(triphenylphosphine)cobalt(I) chloride ((PPh$_3$)$_3$CoCl) as a Co$^1$ source (Scheme 4). However, reactions using this synthesis route were rarely reproducible and often contained a mixture of products, as evidenced by analysis of collected NMR spectra. A representative spectrum for the cleanest reaction using this route is shown in supplementary Figure
S3. Furthermore, synthesis of (PPh$_3$)$_3$CoCl is tricky (see methods) and it is difficult to remove PPh$_3$ from products of reactions with this starting material.

**Scheme 4.** Synthesis route to proposed Co(I)(SNS$_{Me}$) complex using (PPh$_3$)$_3$CoCl as a starting material.

In designing a new and cleaner synthetic route, the anionic (+)SNS$_{Me}$ was isolated as a way to reduce one variable in the one pot synthesis shown in Scheme 4. Furthermore, synthesis and isolation of the anionic (+)SNS$_{Me}$ ligand reveals insight into the formation of SNS$_{Me}$-metal complexes. Baker *et al.* reported that the isolated S$^{Me}$N$^{HS}$ ligand was present only in its cyclic form, as evidenced by NMR spectroscopy (Baker *et al.* 2016, Supplemental Figure 2).$^{21}$ However, the reported iron-SNS$_{Me}$ complexes exhibited a ring-opened tridentate form of the SNS$_{Me}$ ligand (Scheme 1). Previously, it was unclear whether the ligand must be in a ring-opened state prior to coordination, or whether coordination to a metal center forced the ring-opening of the S$^{Me}$N$^{HS}$ ligand. Deprotonation of the ligand results in an anionic nitrogen, which should lead to ring opening to delocalize the negative charge onto the ‘softer’ and larger sulfur atom (Scheme 5).

**Scheme 5.** Mechanism showing S$^{Me}$N$^{HS}$ ring opening upon deprotonation.
Isolation of an anionic thiolate bridged \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) lithium dimer 1 suggests that (a) ring-opening occurs prior to coordination, and (b) ring-opening of the ligand prior to coordination may be necessary to form metal-S\( \text{S}^{\text{Me}} \) complexes.

The anionic \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) lithium dimer 1, shown in Figure 3a and Scheme 3, was obtained by treatment of the \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) ligand with stoichiometric LiHMDS in THF at room temperature. The reaction was almost instantaneous, but required slightly longer times for more concentrated mixtures. The product \([\text{S}^{\text{Me}} \text{N} \text{H} \text{S}]\text{[Li(THF)]}_2\) (1) is dark-red in THF but crystallizes as an orange solid, or dries to a yellow powder upon complete removal of solvent in vacuo, and appears to be stable indefinitely either in solution or as a solid.

\[ \begin{align*}
\text{SMe} & \text{N} \text{H} \text{S} \\
& \text{LiHMDS} \\
& \text{R.T., THF, 30 min} \\
\text{SMe} & \text{N} \text{H} \text{S} \\
& \text{THF} \\
& \text{THF} \\
& \text{1}
\end{align*} \]

**Scheme 6.** Reaction of the \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) ligand with LiHMDS forms the anionic \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) lithium THF-adduct bridged by thiolates.

The \(^1\text{H} \text{NMR} \) spectrum of crystallized 1 showed three equivalents of THF to two equivalents of deprotonated \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) (Figure 7). This was confirmed by X-ray crystallography; the structure of 1 showed one THF molecule per lithium atom and one THF in the outer sphere (Figure 8). The isolation of this lithium \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) dimer confirmed that deprotonation of the \( \text{S}^{\text{Me}} \text{N} \text{H} \text{S} \) ligand in the presence of base results in ring-opening to form the imine-thiolate ligand isomer.
Figure 7. $^1$H NMR spectrum (400 MHz, CD$_2$Cl$_2$) of ring opened ligand, {[SNS$^{Me}$][Li]THF$_2$}$_2$•THF. *denotes CD$_2$Cl$_2$. 
Figure 8. ORTEP diagram of \{{{\text{SNS}}}^\text{Me}{\text{[Li]THF}}_2\}_2^\text{THF}. Oxygen, nitrogen, sulfur, and lithium atoms are labeled with their respective element symbol for clarity. The structure was solved by Dr. Mark Bezpalco in conjunction with Professor Bruce Foxman at the X-ray facility at Brandeis University.

Reaction of two equivalents of 1 with cobalt(II) chloride in the presence of a slight excess of zinc (1-1.25 molar equivalents) yielded the imine-coupled N$_2$S$_2$ cobalt(III) complex
[Co(SNS^Me)_2][(THF)_3Li]_2(µ-Cl)] (2) as a dark-blue solid (Scheme 7).

**Scheme 7.** Reaction of 1 with CoCl$_2$ in the presence of excess zinc to form the N$_2$S$_2$ square planar cobalt(III) complex 2.

The reaction conditions for formation of 2 were very specific, as shown in Scheme 7. Cobalt(II) chloride did not react with the S^MeN^H^S ligand without any base or with 1 in the absence of zinc. Adding the reagents in a different order also resulted in incomplete reactions. For example, CoCl$_2$ was stirred overnight with the S^MeN^H^S ligand and 0.5 equivalents of zinc in THF (yielding no reaction), and then LiHMDS was added and stirred again overnight. This procedure resulted in a mix of products, with some 2 present in addition to many unidentifiable products. Finally, reaction of CoCl$_2$ with 1 in the presence of a large excess of zinc (>5 equivalents) resulted in a similar mix of unidentifiable products. A slight excess of zinc produces a clean synthesis of 2: it was determined that between 1 and 1.25 equivalents yielded optimal synthesis of 2 (slightly more than two times the stoichiometric amount required for a one electron reduction). The slight excess required is likely due to the heterogeneous reduction conditions and the possible impurity of zinc powder reagents.

The specificity of the reaction conditions to form 2 suggests that not only does ring-opening of the ligand occur nearly instantaneously in the presence of base, it also occurs faster than coordination to a metal center and may be necessary for coordination to a cobalt(II) center. Furthermore, formation of 2 requires addition of a reducing agent, such as zinc powder. It is
possible that this reduction plays a role in the imine-coupling of the anionic $^{(-)}$SNS$^{Me}$ ligand to form the N$_2$S$_2$ ligand displayed in 2; however, investigation into the mechanistic details of this coupling reaction is beyond the scope of this thesis.

Scheme 8. Control reactions of CoCl$_2$ with the S$^{Me}$N$^H$S ligand and the anionic ligand 1 under a variety of conditions yielded a less optimal synthetic route to a Co-SNS$^{Me}$ complex than that of Scheme 4.

The $^1$H NMR spectrum for 2 exhibited a wide range of paramagnetic peaks from 13.5 to -103.1 ppm, which is indicative of a high-spin cobalt complex (Figure 9). Only 9 peaks were visible in the spectrum; it is likely that the 10$^{th}$ equivalent set of protons were the methine protons on the newly formed carbon-carbon bond and that these protons were not visible due to their proximity to the paramagnetic cobalt center.
Evans’ $^1$H NMR spectroscopy method returned a magnetic moment measurement of 5.09 µB, which is very close to the value for four unpaired electrons (4.90 µB), and indicates a high-spin complex. However, based on geometry and conventional splitting patterns, the solution state magnetic moment does not agree with the crystal structure of 2: $[\text{Co(SNS}^{\text{Me}}\text{)}_2]^-$ crystallizes as a
square planar complex with the counter-ion \{[(\text{THF})_3\text{Li}]_2(\mu-\text{Cl})]\}^+ (Figure 10). The two thiolate sulfurs and two nitrogen donors are all anionic X-type donors: this information, combined with the overall negative change, suggests that [Co(SNSMe)_2][(\text{THF})_3\text{Li}]_2(\mu-\text{Cl})] contains a cobalt(III) center. This is interesting considering the reductive conditions forming the complex, but can be rationalized by considering the two-electron reduction required for the imine coupling of the ligand.

\[ \text{Figure 10. ORTEP diagrams of [Co(SNSMe)_2][(\text{THF})_3\text{Li}]_2(\mu-\text{Cl})]. Cobalt, nitrogen, and sulfur atoms are labeled with their respective element symbol for clarity. (a) Structure of a single [Co(SNSMe)_2] anion, with counterion omitted for clarity. (b) Structure of the asymmetric unit, which includes two [Co(SNSMe)_2][(THF)_3\text{Li}]_2(\mu-\text{Cl})]. The crystals were obtained by Dr. Cassandra Hayes at the University of Rochester and verified by NMR to be the same species as consistently obtained in the reaction of the ring-opened deprotonated ligand \{[SNSMe][\text{Li}][\text{THF}]_2\}_2\text{THF} with \text{CoCl}_2. The structure was solved by William J. Brennessel at the X-ray Crystallographic Facility at the University of Rochester.} \]

A similar imine coupling of aminobenzenethiolate ligands forming \(\text{N}_2\text{S}_2\) complexes in the presence of cobalt and iron metals has been reported and studied by Wieghardt et al.\(^{23-24}\)

Wieghardt and Sproules et al showed that 2,3-dihydro-2-phenylbenzothiazole coupled in the
presence of Co\(^{2+}\) via a new carbon-carbon bond formed between the imine carbons (Scheme 9).\(^{23}\) The resulting Co(N\(_2\)S\(_2\)) \(3\) was then reduced with cobaltocene to give the Co\(^{III}\)(N\(_2\)S\(_2\)) anion \(4\) shown in Scheme 9.\(^{23}\) This anionic Co\(^{III}\)(N\(_2\)S\(_2\)) complex is very similar to the observed complex \(2\) described in this thesis, and so a brief comparison of the structures follows.

\[\text{Scheme 9. Coordination and imine coupling of 2,3-dihydro-2-phenylbenzothiazole in the presence of cobalt(II) under reflux described by Wieghardt et al.}^{23}\]

As shown in Figure 10, there are two molecules in the asymmetric unit of the crystallized Co\(^{III}\)(N\(_2\)S\(_2\)) complex \(2\); however, their bond lengths and angles are nearly identical (bond lengths deviate by at the most 0.004 Å, and angles by 0.7°). Both contain a slightly distorted square planar cobalt-N\(_2\)S\(_2\) center: around the cobalt center, the average S-Co-S bond is 97.7° whereas the average N-Co-S and N-Co-N bonds are 88.5° and 85.5° respectively (Table ). Sproules et al report a planar square planar cobalt center for complex \(4\), with a dihedral Co-NS angles of 2.2°. Complex \(2\) shows a slightly less perfectly planar center, as the N-Co-S bonds across the plane are not quite linear, at 173.7° (data not shown). Furthermore, Sproules et al reported the phenyl substituents in complex \(4\) to be almost trans to the cobalt(N\(_2\)S\(_2\)) plane, at (on average) 83.4°.\(^{23}\) In complex \(2\) the aryl-SMe groups are not quite perpendicular to the cobalt-N\(_2\)S\(_2\) plane, with an average N-C-C(aryl) bond angle of 111.8°; however, they are almost trans to each other, pointing in opposite directions from the Co-N\(_2\)S\(_2\) plane. The average Co-N and Co-2 bonds are 1.83 Å and 2.17 Å, respectively: this is longer than the distances reported by Sproules et al, which were 1.38 Å and 1.76 Å, respectively.\(^{23}\)
Cyclic voltammetry (CV) measurements of 2 show three redox processes (Figure 11), with two fully reversible reductions at -0.13 V and -0.61 V (Figure 12), and one quasi-reversible reduction at -1.85 V. This further confirms that [Co(SNSMe₂)_2][(THF)_3Li][μ-Cl] has potential for multi-electron redox catalysis. The milder reductions may correspond to Co^{III}/Co^{II} and Co^{II}/Co^{I} redox couples. However, it is also possible that these redox processes correspond to ligand-based reduction events, especially considering that they occur at such mild potentials. The partially irreversible reduction likely forms an unstable form of the complex that is no longer able to be re-oxidized, either resulting in decomposition or ligand dissociation.

<table>
<thead>
<tr>
<th>Atoms</th>
<th>Average Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Co-S</td>
<td>97.7</td>
</tr>
<tr>
<td>N-Co-S</td>
<td>88.5</td>
</tr>
<tr>
<td>N-Co-N</td>
<td>85.5</td>
</tr>
<tr>
<td>N-C-C</td>
<td>111.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bond</th>
<th>Average Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-N</td>
<td>1.830</td>
</tr>
<tr>
<td>Co-S</td>
<td>2.173</td>
</tr>
</tbody>
</table>

Table 1. Average bond angles around the cobalt metal center in complex 2.

Table 2. Average bond lengths for Co-N and Co-S bonds for complex 2.
Figure 11. Cyclic voltammogram of [Co(SNS\text{Me})$_2$][(THF)$_3$Li]$_2$(µ-Cl)] dissolved in 0.4 M TBA-PF$_6$•THF, scanned from -3.25 V to 0 V at 0.1 V s$^{-1}$, reported versus Fe/Fe$^+$. Open circuit potential: 0.12 V.
Figure 12. Cyclic voltammogram of [Co(SNSMe)2][(THF)2Li]2(μ-Cl)] dissolved in 0.4M TBA-PF6•THF, scanned from -1.25 V to 0.35 V at 0.1 V s⁻¹, reported versus Fc/Fc⁺. Open circuit potential: -1.13V.

Although Sproules et al did not report a CV spectrum for complex 4, their CV spectrum recorded for complex 3 roughly matches that which we report here for complex 2, with two fully reversible redox process (although they report one reduction and one oxidation), and one quasi-reversible reduction. Sproules et al discuss in depth the redox active and non-innocent character of the N₂S₂ complex, and in fact conclude that it is difficult to label either complex 3 or 4 as Co³⁺ (however, they do suggest based on their electronic measurements and density functional theory calculations that both complexes have more Co³⁺ character). In the same way, although we suggest based on the crystal structure obtained that complex 2 is Co³⁺, it is difficult to unambiguously assign an oxidation state to this novel Co(N₂S₂) complex. Similarly, it is quite possible that some of the redox processes observed in the CV measurements were ligand-based, and at this point we do not have enough information to definitively assign either the redox
processes or the oxidation state to the ligand or to the metal. Future electronic measurements and density functional theory calculations will help to further assign the nature of the oxidation state and redox behavior of complex 2.

Materials and Methods

General

Air and moisture sensitive procedures were carried out either inside an N₂ atmosphere glovebox or using a standard Schlenk line equipped with N₂ gas and vacuum. All glassware was oven dried for two or more hours. Solvents for air and moisture sensitive reactions were degassed under argon, dried using a Seca Solvent System by Glass Contour, and stored over 3 Å molecular sieves under N₂ atmosphere. The SN₇HS₇Me ligand and (PPh₃)₃CoCl were prepared according to modified literature procedures, as described below; all other starting materials were purchased from Sigma Aldrich, Strem Chemicals, or Alfa Aesar. Acquisition of ¹H and ³¹P NMR spectra was conducted on a Varian 400 MHz spectrometer. Chemical shifts are reported in δ (referenced to residual solvent peaks for ¹H spectra), with coupling constants reported in Hz with multiplicities denoted as s (singlet), d (doublet, or dd for doublet of doublets, etc.), and m (multiplet). Chemical shift only is reported for ¹H paramagnetic resonances.

Synthesis

Preparation of SN₇HS₇Me ligand:

The SN₇HS₇Me ligand was prepared according to Baker et al., with some slight procedural modifications described here. In the glovebox, ethanol (20 mL) and aminothiophenol (1.4 mL, 12 mmol) were combined in an oven-dried 100 mL Schlenk flask. The flask was then transferred to the Schlenk line, and 2-methylthiobenzaldehyde (1.5 mL, 11 mmol) was added via a syringe under positive N₂ pressure, giving a pale yellow solution. Stirring for 24 hours at room
temperature under N\textsubscript{2} flow produced a white precipitate. The white solid was allowed to continue precipitating in the freezer for 20 minutes, collected on a medium frit, and then washed with cold dry ethanol (200 proof ethanol, chilled in the freezer over 3 Å molecular sieves). Drying \textit{in vacuo} overnight yielded a fluffy white powder (2.58 g, 83\%), which was stored in the glovebox. \textsuperscript{1}H NMR (400 MHz, C\textsubscript{6}D\textsubscript{6}) \(\delta\) 1.96 (s, 3H, S-CH\textsubscript{3}), 3.37 (br s, 1H, N-H), 6.37 (d, \(J = 8\) Hz, 1H, aryl), 6.61 (ddd, \(J = 7.4\) Hz, 7.4 Hz, 1.2 Hz, 1H, aryl), 6.65 (d, \(J = 3.6\)Hz, 1H, aryl), 6.83 (dd, \(J = 8\) Hz, 1.2 Hz, 2H, aryl), 6.87 (dd, \(J = 7.6\) Hz, 1.6 Hz, 1H, aryl), 6.89 – 6.97 (m, 2H, aryl), 7.70 (d, \(J = 3.6\) Hz, 1H, methine C-H).

\textit{Preparation of “Super-dry” ethanol:}

Procedures were drawn from the literature (see \textit{Purification of Laboratory Chemicals}).\textsuperscript{25} A 1 liter Schlenk flask was fitted with a reflux condenser and stir bar. Under an N\textsubscript{2} atmosphere, magnesium turnings (2.5 g, washed, see below) were activated with iodine (I\textsubscript{2} crystals, 0.25 g, pure, desiccated): the flask was heated using a heat gun until the iodine vapors filled the flask and coated the magnesium turnings. After cooling to room temperature, ethanol (50 mL) was added and the mixture was refluxed for 30 minutes, using a heating mantle and a sand bath. After cooling again, more iodine was added (I\textsubscript{2} crystals, 0.21 g). Refluxing for 2 hours at 110ºC resulted in a white suspension, with a white precipitate. The reflux condenser was replaced by a distillation apparatus connected to a Strauss flask, and collection of the distillate at 78ºC yielded “super-dry”\textsuperscript{25} ethanol. Inside the glovebox, the ethanol was decanted and stored in a bottle with 3 Å activated molecular sieves.

\textit{Preparation of Magnesium turnings:}

Magnesium turnings (2.5 g) were washed with absolute ethanol (~10 mL) and ether (2 x 15 mL), and then heated at 129ºC and dried \textit{in vacuo} for 2 days.
Preparation of \((\text{PPh}_3)_3\text{CoCl}\):

Several methods are reported in the literature for preparation of tris(triphenylphosphine)cobalt(I) chloride \((\text{PPh}_3)_3\text{CoCl}\). Having tried over half a dozen (see below), reported here for reference is the most straightforward procedure with slight modifications, which produces moderate yield. This procedure was drawn from a paper published by the Fout group in 2015.\(^{26}\)

Cobalt(II) chloride (0.3272 g, 2.52 mmol) was weighed out under an N\(_2\) atmosphere. An oven-dried round-bottom flask was brought under an N\(_2\) atmosphere. The cobalt chloride was added under an N\(_2\) flow, and then the flask was capped with a septum. 25 mL of ethanol (200 proof) was degassed with N\(_2\) in a separate round bottom flask, and was then added via cannula transfer to the reaction flask. Triphenylphosphine (2.00 g, 7.63 mmol) was added under a positive flow of N\(_2\) gas, and the flask was fitted with a reflux condenser capped with a septum. The mixture was heated to 70\(^\circ\)C under a constant flow of N\(_2\) gas, resulting in a light-blue suspension. The flask was then removed from heating, and NaBH\(_4\) (0.080 g, 2.1 mmol) was added under a flow of N\(_2\) gas. Upon addition of NaBH\(_4\), the mixture turned instantly green-brown. Evolution of hydrogen gas was visible, and the mixture was stirred until the flask was cooled to room temperature. The resulting green-brown solids were collected on a Buchner funnel and washed with cold ethanol until the brown color disappeared. Washing with deionized water (5 mL), ethanol (10 mL), and hexanes (30 mL) left a dark green solid. Drying overnight \textit{in vacuo} gave a forest green powder confirmed to be \((\text{PPh}_3)_3\text{CoCl}\) by \textsuperscript{31}P and \textsuperscript{1}H NMR (1.143 g, 61\%). This moderate yield is slightly lower than that reported by the Fout group (86\%), but is justified by the less tedious procedure.\(^{26}\)
Additional procedures for preparation of (PPh$_3$)$_3$CoCl can be found in the literature. Often, they suggest isolation of the (PPh$_3$)$_2$CoCl$_2$ intermediate or lengthy Schlenk line filtration and are usually inconsistent in yield.\textsuperscript{27-30} Fortunately, the above method is effective because (PPh$_3$)$_3$CoCl is slightly air stable, and so workup can be conducted on the bench in air.

Selected NMR spectra and synthesis for proposed [SNS$_\text{Me}$]Co(PPh$_3$):

(PPh$_3$)$_3$CoCl (0.3090 g, 0.35 mmol) and SN$_\text{H}$S$_\text{Me}$ ligand (0.0910 g, 0.35 mmol) were combined as solids in a 20 mL vial with a stir bar. Benzene (~10 mL) was added, and the reaction immediately began to turn a dark blue. The reaction was stirred for 30 minutes, and then cooled at -35ºC overnight. The solvent was removed \textit{in vacuo} and the resulting dark blue product was washed with Et$_2$O (2 x 1 mL) to remove any remaining starting material (visible as a green suspension in Et$_2$O). The solids were then washed with benzene (2 x 3 mL), and filtered through Celite, removing the triphenylphosphine by-product. The remaining solid product was dissolved in dichloromethane (5 mL), and dried \textit{in vacuo} to yield a dark burgundy solid. $^1$H NMR (400 MHz, CD$_2$Cl$_2$) δ -4.41 (s, br), -3.53 (s, br), -3.01 (s, br), 6.05 (s), 6.14 (s, br), 6.33 (s, br), 6.87 (s), 6.89-7.03 (m), 13.78 (s, br), 15.48 (s, br). No peaks were observed in the $^{31}$P NMR spectrum.

Synthesis of [{[SNS$_\text{Me}$][Li(THF)]}$_2$•THF:

The SN$_\text{H}$S$_\text{Me}$ ligand (0.1809 g, 0.70 mmol) and lithium hexamethyldisilazide (LiHMDS, 0.1167 g, 0.70 mmol) were combined as solids in a 20 mL vial with a stir bar. Approximately 8 mL of THF was added, and the solution immediately turned dark red. The reaction mixture was stirred for 30 minutes and then filtered through Celite. The Celite was washed with THF (2 x 1 mL) until no red color came through with the filtrate. THF was removed \textit{in vacuo}, yielding a yellow-orange powder (0.2225 g, 89%). $^1$H NMR (400 MHz, CD$_2$Cl$_2$) δ 1.60 (s, 12H, THF), 2.45
(s, 6H, S-CH₃), 3.55 (s, 12H, THF), 6.79 (d, J = 3.6 Hz, 4H, aryl), 6.85 (ddd, 2H, 4 Hz, 3.6 Hz, 3.6 Hz, aryl), 7.14 – 7.22 (m, 4H, aryl), 7.34 – 7.43 (m, 4H, aryl) 8.04 (d, J = 6.8 Hz, 2H, aryl), 8.75 (s, 2H, N-CH). X-ray quality crystals were grown from THF at -35°C and the structure was solved by Dr. Mark Bezpalko and Professor Bruce Foxman at Brandeis University (Figure 8).

Note that the reaction mixture can be stirred longer (overnight) with equally good results.

Furthermore, at high concentrations and cold temperatures, the product will precipitate or crystallize out of THF.

**Synthesis of [Co(SNSMe₂)₂][(THF)₃Li]₂(µ-Cl)]:**

Cobalt chloride (0.0079 g, 0.06 mmol), the deprotonated ligand {[SNSMe][Li(THF)]₂} (0.0411 g, 0.06 mmol), and excess zinc (0.0055 g, 0.08 mmol) were added together as solids in a 20 mL vial. THF (5 mL) was added and the mixture was cooled in the freezer for 30 minutes. The reaction mixture was allowed to warm to room temperature and was then stirred overnight. The resulting dark blue-black suspension was filtered through Celite, and washed with THF (3 x 1 mL) until no blue color came through with the filtrate, leaving only gray solids on the Celite plug. The THF was removed from the filtrate *in vacuo*, yielding a dark flaky blue solid. ¹H NMR (400 MHz, CD₃CN) δ -103.07 (s, 2H), -97.0 (s, 2H), -20.07 (s, 2H), -1.93 (s, 2H), 1.82 (s, THF-CH₂-CH₂-CH₂), 2.62 (s, 2H), 2.97 (s, 6H), 3.67 (THF-O-CH₂), 7.64 (s, 2H), 11.88 (d, 2H, J = 7.2 Hz), 13.52 (s, 2H). Crystals of a compound with matching NMR resonances were grown by Dr. Cassandra Hayes at the University of Rochester, and the structure is shown in Figure 10.

**Measurement of Solution State Magnetic Moment: Evan’s Method NMR:**

[Co(SNSMe₂)₂][(THF)₃Li]₂(µ-Cl) (0.0040 g) was dissolved in CD₃CN (0.3475 g, 0.4117 mL) and pipetted into an NMR tube containing a capillary of CD₂Cl₂ doped with protonated
dichloromethane. The chemical shift of the residual solvent signal for the pure capillary sample of CD$_2$Cl$_2$ (6.210 ppm) was compared to that of the trace CH$_2$Cl$_2$ impurity in the paramagnetic sample dissolved in CD$_3$CN (5.448 ppm). This information was used to calculate the solution state magnetic moment, as follows:

\[
X_M = \frac{477(\Delta Hz)}{Q * V_1 * c} = \frac{477[(6.2100 ppm - 5.4478 ppm) \frac{400 MHz}{ppm}]}{0.0040 g \frac{575.69 g}{mol}} = -1.08 \times 10^{-2} \frac{emul}{mol}
\]

\[
\mu_{eff} = \sqrt{8X_M * 298} = \sqrt{8(-1.08 \times 10^{-2}) * 298} = 5.09 \mu B
\]

This measurement is close to the theoretical value obtained for four unpaired electrons \((S = 2)\):

\[
\mu_s = 2.00\sqrt{S(S + 1)} = 2.00\sqrt{2(2 + 1)} = 4.90 \mu B
\]

**Cyclic Voltammetry:**

Measurements of [Co(SNS$^{Me}_2$)][(THF)$_3$Li]$_2$(µ-Cl)] were conducted under an N$_2$ atmosphere in a standard single-compartment cell with a glassy carbon working electrode, platinum auxiliary electrode, and silver pseudoreference electrode. [Co(SNS$^{Me}_2$)][(THF)$_3$Li]$_2$(µ-Cl)] (10.5 mg) was dissolved in approximately 3 mL of a 0.4 M electrolyte solution of tetrabutylammonium hexafluorophosphate (TBA-PF$_6$) dissolved in THF. Potentials are reported referenced to ferrocene/ferrocenium.

**UV-vis spectrum of [Co(SNS$^{Me}_2$)][(THF)$_3$Li]$_2$(µ-Cl)]:**

Under an N$_2$ atmosphere, [Co(SNS$^{Me}_2$)][(THF)$_3$Li]$_2$(µ-Cl)] (0.0060 g, 0.01 mmol) was dissolved in 10.4 mL of THF to make a 1 mM solution. This solution was sequentially diluted in THF to produce 4 mL of a 0.1 mM solution, and 3 mL of a 0.01 mM solution. Three airtight
quartz cuvettes were brought in the glovebox, and were each filled with 3 mL of respectively pure THF, 0.1 mM [Co(SNSMe)2][(THF)3Li]2(µ-Cl), and 0.01 mM [Co(SNSMe)2][(THF)3Li]2(µ-Cl)]. UV-visible spectra were recorded using the program “Scan” on a Cary 50 UV–vis spectrometer from 200 - 1000 nm.

**Conclusion**

This work reports the first example of a Co-SNS complex for NH3BH3 dehydrogenation, and the third example of a homogenous cobalt catalyst for NH3BH3 dehydrogenation, breaking new ground in poly-dentate 1st row complexes for H2 fuel catalysis. Isolation of the anionic \(^{(−)}\text{SNS}^{\text{Me}}\) 1 was achieved, characterizing a novel \([[\text{SNS}^{\text{Me}}][\text{Li(THF)}]]_2\cdot\text{THF}\) by crystallographic structure determination and NMR spectroscopy. The isolated dimer showed a ring-opened form of the anionic ligand, and confirmed that the SMeNH3 ligand reported by Baker et al. undergoes ring-opening in the presence of a base. A novel cobalt(III) complex was synthesized using the anionic \(^{(−)}\text{SNS}^{\text{Me}}\) ligand 1, revealing coordination of a new imine-coupled N2S2 ligand to CoIII in a square planar geometry. The new cobalt complex 2 showed multiple reversible redox couples accessible at mild potentials, indicating its potential for catalysis of multi-electron redox processes.

Co-SNS complexes have potential for dehydrogenation and hydrogenation catalysis, as evidenced by the dehydrogenation of ammonia borane effected by the proposed \((\text{SNS}^{\text{Me}})\text{Co}^{1}(\text{dmpe})\) complex. Furthermore, the CoIII(N2S2) complex described here could have potential beyond simple NH3BH3 dehydrogenation, and future studies should investigate the catalytic range of this new complex. Beyond the novel complex described here, the high yielding synthesis of the SMeNH3 ligand reported by Baker et al and the straightforward and clean,
stoichiometric synthesis of the Co$^{3+}$(N$_2$S$_2$) complex make this an ideal platform for developing a whole series of new Co-SNS complexes for hydrogenation and dehydrogenation catalysis.

Supplemental Spectra:

Figure S1. $^{11}$B NMR (96 MHz) spectra showing of ammonia borane dehydrogenation catalysis at room temperature using proposed [SNS$_{Me}$]Co$^0$(dmpe).
Figure S2. $^1$H NMR spectrum (400 MHz, C$_6$D$_6$) of SN$^{H/SMe}$ ligand. *denotes NMR solvent.
Figure S3. $^1$H NMR spectrum (400 MHz, CD$_2$Cl$_2$) of proposed [SNS$^{Me}$][PPh$_3$Co(I)]. *denotes CD$_2$Cl$_2$; † denotes solvent impurities benzene, toluene, and pentane.
**Figure S4.** UV-vis spectrum of [Co(SNSMe)₂][(THF)₃Li]₂(μ-Cl)] (1 x 10⁻⁴ M in THF): molar extinction coefficient plotted against wavelength (nm). This graph was obtained from absorbance data using the Beer-Lambert law: $A = εcλ$.

**References**

ABSTRACT

Chapter 2. Degradation of Organophosphonates: Study and Characterization of a Novel Fusion Diiron Oxygenase from *F. multimorphosa*.

A thesis presented to the Department of Biochemistry
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The HD-domain superfamily describes enzymes that are designated as phosphohydrolases; however, recent discoveries have shown that two HD-domain proteins, *myo*-inositol oxygenase (MIOX) and PhnZ, function as oxygenases. Both MIOX and PhnZ contain a diiron cluster that performs a novel 4 electron oxidative cleavage of, respectively, carbon-carbon or carbon-phosphorous bonds via incorporation of O$_2$. In conjunction with the enzyme PhnY, the HD-domain protein PhnZ catalyzes the degradation of 2-aminoethylphosphonate to glycine and inorganic phosphate, showing us a new strategy to cleave an ‘old’ bond.

This work confirms a third known example of an HD-domain diiron oxygenase, and shows that the fusion oxygenase obtained from *F. multimorphosa* roughly resembles the catalytic action of the PhnY/Z system. The fusion oxygenase has a wider range of phosphonate substrates than the PhnY/Z system and a wider range of pH stability, as perhaps expected for a pathogenic fungus that must be adaptable to environmental niches. We have now defined and assigned function to a novel fusion protein, and in the process showed that the fusion protein is perhaps a strategy to chemically diversify and expand the functional repertoire of these enzymes.
Introduction

The recently defined HD superfamily of proteins is characterized by a conserved motif of four charged amino acids (H …. HD …. D) that coordinate a metal cation in the active site of the enzyme.\textsuperscript{1-2} HD-domain proteins make up over 130,000 proteins arranged in different domain architectures.\textsuperscript{3-4} Although HD-domain proteins have been assumed to fill a diverse range of functions, including phosphohydrolase and nuclease activity, the catalytic scope and mechanism of these enzymes are still being explored.\textsuperscript{1-2} HD-domain enzymes have been postulated to be phosphohydrolases\textsuperscript{2}; however, two known enzymes employ dioxygen to oxidatively convert their substrates.\textsuperscript{3-7} These enzymes are myo-inositol oxygenase and the organophosphonate cleaving enzyme PhnZ.\textsuperscript{3-7}

PhnZ is found in a two-enzyme pathway together with the preceding enzyme in the pathway, PhnY.\textsuperscript{3-4} PhnY is a non-heme Fe- and $\alpha$-KG-dependent oxygenase that acts on 2-aminoethylphosphonate (2-AEP) and installs a hydroxyl group in the C1 position, creating the 1-hydroxy-2-aminoethylphosphonate (OH-AEP).\textsuperscript{3-4} PhnZ is an HD-domain Fe-dependent enzyme that uses molecular oxygen as a co-substrate to afford the 4e- cleavage of the C-P bond of OH-AEP, yielding phosphate and glycine as coproducts. Previously established routes for C-P bond cleavage were variations on hydrolase activity, and so the discovery of the oxygenase activity of PhnZ revealed an entire new enzyme mechanism for cleaving a C-P bond.\textsuperscript{3-6} To date there is no structure of PhnY (which is very selective on the substrate it acts on), and only a structure of PhnZ (which is more promiscuous in the phosphonate it can cleave). \textsuperscript{3-5}
Scheme 10. PhnY hydroxylates 2-AEP to produce OH-AEP, which is broken down into glycine and phosphate by PhnZ. Adapted from Wörsdörfer et al 2013.3

Crystal structures and characterization of PhnZ revealed that it binds two iron atoms in a mixed-valent form to catalyze C-P bond cleavage of OH-AEP.3,6 Recent research has continued to define the mechanism for the oxidative cleavage effected by PhnZ, and current models suggest the C-P bond cleavage step occurs via an oxygen insertion during a heterolytic cleavage of an O-O bond.5 Further study into the mechanism of other prospective HD oxygenases in addition to PhnZ, could provide insight as to the evolutionary significance of HD oxygenases and the oxidative cleavage of C-P bonds.3-6

Biochemical characterization of a novel fungal metalloenzyme is presented, which represents a fusion between two different domains, both harboring distinct, but Fe-dependent metallocofactors. It consists of a N-terminal domain, which contains a monoiron α-KG dependent oxygenase binding motif and the C-terminal domain, which harbors an HD-binding motif characteristic of dimetal (and diiron) enzymes1-4, that has a significant homology to that of the HD-domain mixed-valent diiron oxygenases (MVDOs). We refer to this enzyme as FeOf, or iron oxo-fusion, suggesting that it is an iron dependent fusion oxygenase consisting of PhnY-like and PhnZ-like domains. The PhnY-like domain has 53% similarity and 33% identity to that of the PhnY protein, whereas the PhnZ-like HD-domain has 50% similarity and 30% identity to that of PhnZ. Inspired by the similarity to the PhnY / PhnZ pair, we hypothesized that the substrate specificity for this fusion enzyme will be imposed by the PhnY-like domain.
The questions that we hope to answer in this study are the following: does the fusion enzyme take the same physiological substrate as PhnY/Z (2-AEP)? Furthermore, is there an advantage or disadvantage to having a coupled system versus an enzyme pair, in terms of substrate specificity and rate of substrate conversion? We report here through screening of various organophosphonates on the expressed and purified FeOf protein from *F. multimorphosa* that not only does this protein take the same substrate as the PhnY/Z system (2-AEP), but that it also can catalyze conversion of an expanded range of substrates.

**Results and Discussion**

Bioinformatic analyses identified a novel uncharacterized fusion HD-domain enzyme from the fungus *Fonsecaea multimorphosa* containing both a PhnY-like (an \(\alpha\)-KG dependent mono-iron oxygenase) and a PhnZ-like (HD-domain diiron oxygenase) domain. Using the I-TASSER server, a structural similarity search was conducted on the pdb database. The best homology model was generated by overlaying the putative mono-iron domain with the \(\alpha\)-KG dependent mono-iron carbocyclase SnoK\(^8\) and the HD-diiron domain with PhnZ.\(^4\) The distance between the putative active sites was 46Å (Figure 13). To determine whether these active sites contained iron, we quantified the iron contained in the natively expressed protein using FerroZine assays and Mössbauer spectroscopy.
Expression of FeOf in *E. Coli* and purification by Ni-NTA chromatography yielded FeOf protein containing 2.1 iron atoms per protein. Assessment of the environment of the iron was carried out using Mössbauer spectroscopy (Figure 14). Most of the signal was taken up by a doublet, which corresponded to two anti-ferromagnetically coupled $S = 5/2$ Fe$^{III}$ atoms. The isomer shift was 0.495 mM s$^{-1}$, with a quadrupole splitting ($\Delta$Eq) of 0.78 mm s$^{-1}$, which
corresponds almost exactly to that reported for PhnZ.\textsuperscript{3-4} Based on the simulated fit, approximately 85\% of the iron present in the protein was in the diiron site.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Mossbauer_spectrum.png}
\caption{Mössbauer spectrum of FeOf protein expressed in M9 minimal media with $^{57}$Fe.}
\end{figure}

Comparison between the stabilities of the FeOf protein and the PhnZ/Y systems was initially carried out via thermal shift assays to determine melting temperatures. It was found that the FeOf protein had two denaturation regions, corresponding to two melting temperatures (Tm’s) for its putative mono-iron and diiron domains. These matched well with the Tm’s obtained for PhnY and PhnZ (Figure 15). However, both Tm’s of the fusion protein (46.3°C and 64.3°C) were higher than that of their respective counterparts in PhnY/Z (37.3°C and 62.8°C, respectively). We hypothesized that the greater stability of the FeOf protein might impart a different activity profile than the PhnY and PhnZ systems, and so a series of multiple turnover assays were carried examining substrate turnover under a variety of different conditions.
Figure 15. Melting curves comparing FeOf, PhnY, and PhnZ proteins. Data for each protein was collected at its optimal pH (e.g. 6.5, 7.3, and 7.3, respectively). The data showed that the FeOf protein has a biphasic denaturation, which corresponds approximately to that of PhnY at lower temperatures, and that of PhnZ at higher temperatures. However, both melting temperatures for the FeOf protein were higher than their corresponding PhnY/Z counterparts, suggesting that the FeOf protein is overall more stable than the PhnY/Z system.

The optimal pH buffer for each enzyme was chosen based on the conditions which yielded the highest melting temperature and thus the greatest stability: respectively, FeOf, PhnY, and PhnZ were determined to be most stable in pH 6.5, 7.3, and 7.3 (data not shown for PhnY and PhnZ). Thermal shift data for the FeOf protein (Figure 16) showed fair stability over a wide range of pH’s, from pH 5 to pH 9. However, screening assays with 2-AEP at the pH’s shown in Figure 16 only yielded substrate turnover at pH 6.5, 7.3, and 8.
Figure 16. Selected melting curves for the FeOf protein at different pH’s. The optimal pH for the protein was pH 6.5; however, it is interesting to note that the putative mono-iron domain is more stable at higher pHs yet cannot tolerate lower pH’s. Curiously, this is the opposite of PhnZ, which can tolerate lower pH’s but not higher pH’s.

The activity of the FeOf protein was compared to that of the PhnY/Z system at pH’s 7.3 and 6.5 to see which enzyme system was more capable of coping with a non-optimal pH (Figure 17). Surprisingly, the FeOf system in its non-optimal pH had no advantage over the PhnY/Z system in its respective non-optimal pH environment. Indeed, the PhnY/Z system was more competent at pH 6.5 than the FeOf protein was at pH 7.3, respectively performing 134 turnovers as compared to 62 turnovers.
Figure 17. FeOf catalyzed conversion of 2-AEP to phosphate (P$_i$) over time measured by $^{31}$P ($^1$H) NMR spectra (121 MHz, 20% D$_2$O), at 25°C. The fraction of P$_i$ was calculated by integrating the resonance peaks for 2-AEP and P$_i$, and dividing the value for P$_i$ by the sum of the two. 2-AEP (2 mM) was incubated for two hours with either protein system (10 $\mu$M) in the presence of Fe$^{2+}$, sodium ascorbate, and $\alpha$-KG for the pH environments shown above.

Furthermore, it was found that the FeOf protein was substantially slower at turning over substrate than the PhnY/Z system, taking 140 minutes to complete 200 turnovers (Figure 18), as compared to 30 minutes for the PhnY/Z system (as reported in the literature). The rate of conversion of 2-AEP to phosphate was measured at different time points for the FeOf protein, establishing an almost linear rate (Figure 18). None of these experiments showed any hydroxylated intermediate, such as might be expected from the putative PhnY-like mono-iron site of the FeOf protein.
Figure 18. FeOf catalyzed conversion of 2-AEP to phosphate (P_i) over time measured by $^{31}$P {$^1$H} NMR spectra (121 MHz, 20% D$_2$O), at 25°C. The fraction of P$_i$ was calculated by integrating the resonance peaks for 2-AEP and P$_i$, and dividing the value for P$_i$ by the sum of the two. 2-AEP (2 mM) was incubated with FeOf protein (10 µM) in the presence of Fe$^{2+}$, sodium ascorbate, and α-KG for the time points shown above. Conversion of 2-AEP to P$_i$ follows a roughly linear trend over time, with 200 turnovers in 140 minutes.

The physiological substrate scope of the FeOf enzyme was further explored by screening a variety of phosphonate substrates (Figure 19). Multiple turnover assays were conducted over 140 minutes for each substrate, and the results were measured by $^{31}$P NMR spectroscopy. The FeOf protein completed 200 turnovers for both 2-AEP and 3-APP, converting both substrates all the way to phosphate, and slightly catalyzed the degradation of TMAEP to phosphate. However, propyl-AEP was not taken as a substrate by the enzyme (Figure 20). The conversion of 3-APP by the FeOf protein was a sign of expanded substrate scope of the fusion enzyme as compared to the PhnY/Z pair, but the preferred substrate for the FeOf protein was still not established.
Figure 19. Structures of different phosphonates used for screening of the physiological substrate for the FeOf fusion protein.
To establish whether 2-AEP or 3-APP was the physiological substrate for the FeOf protein, 1 mM of each substrate was incubated simultaneously for 1 hour with 10 μM of the FeOf protein in a competition assay. Product formation was measured by $^{31}$P NMR, showing that the FeOf protein preferentially converted 2-AEP over 3-APP, as evidenced by the remaining 3-APP starting material (Figure 21).

The conversion of 3-APP to phosphate and slight conversion of TMAEP to phosphate by the fusion enzyme is exciting because the PhnY/Z system has been documented as very selective in its substrate scope. Further exploration comparing the substrate selectivity of the FeOf and PhnY/Z systems was examined by incubating 3-APP with each protein system for two hours in a multiple turnover assay (Figure 22). In two hours, the FeOf protein completed 120 turnovers, as compared to the PhnY/Z system, which completed 72 turnovers.

Furthermore, the data from the catalytic conversion of 3-APP by the FeOf protein showed a small amount of hydroxylated substrate. Because no hydroxylated substrate was observed in
any experiment with 2-AEP catalyzed by the fusion protein, and because most of the iron in the FeOf protein exists in the diiron site, accumulation of the hydroxylated substrate requires that the HD domain rate was slowed down by the foreign substrate relative to the putative mono-iron domain. This could suggest the HD domain of the FeOf protein has greater substrate selectivity than its putative mono-iron domain. If this were true, it would be the opposite of the PhnY/Z case, where substrate specificity is imposed by the PhnY domain.³

![Figure 22](image)

**Figure 22.** ³¹P {¹H} NMR spectra (121 MHz, 20% D₂O), at 25°C comparing activity of the FeOf protein versus that of the PhnY/Z system using 3-APP as a substrate. Reactions were run for 120 minutes with 10 μM protein (in the case of PhnY/Z, 10 μM of each), and 2 mM substrate in the presence of Fe²⁺, sodium ascorbate, and α-KG (a) PhnY/Z system showed 72 turnovers of the 3-APP substrate to inorganic phosphate (marked P₁). The reaction was run at pH 7.3 (b) FeOf protein showed faster conversion of conversion of 3-APP to inorganic phosphate, with 120 turnovers. The hydroxylated intermediate (OH-APP) was also observed.

Since the OH-AEP was never observed in any experiments incubating 2-AEP with the FeOf protein, stopped-flow kinetic experiments were run to determine the Kd of the diiron site. A typical stop-flow set-up is shown in Figure 23. In the experiment, the protein and sample were loaded onto respective syringes, and rapidly mixed in an IR cell while monitoring by absorption spectroscopy. All wavelengths were monitored using a photo-diode array, and the resulting traces could then be examined to look for minima and maxima corresponding to changes in
absorbance resulting from binding at specific wavelengths (e.g. Figure 24). Measuring the changes in absorbance corresponding to binding yielded an exponential growth or decay curve, which was then fit to extract Kobs for a given substrate concentration. An example of such a trace is shown in Figure 25, for 500 μM OH-AEP, yielding a Kobs of 19.95 ± 0.43 mM s⁻¹.

**Figure 23.** Set up for stopped-flow kinetics experiments. (a) FeOf protein was mixed with various concentrations of OH-AEP to extrapolate Kobs at different substrate concentrations. (b) FeOf protein premixed with an excess of OH-AEP (>2 mM) was mixed rapidly with oxygenated buffer in a single turnover assay.
Figure 24. Stop-flow absorption spectra showing the binding of OH-AEP to the HD-domain of the FeOf protein. FeOf protein incubated with 1mM OH-AEP over 20 seconds. Pictured are eight representative time traces collected from the incubation of 150 µM FeOf protein with 1 mM of OH-AEP substrate. (a) Panel A shows the relative absorbance collected from a photo-diode array at wavelengths 240-720 nm. (b) Panel B shows a difference spectrum with the change in absorbance versus wavelength constructed subtracting the spectrum at time zero from all eight representative traces.

Figure 25. Stopped-flow kinetic trace of the binding of 500 µM OH-AEP to 75 µM FeOf protein showing relative absorbance at 399 nm over time. The increase in relative absorbance over time shows the binding of OH-AEP to the FeOf diiron active site.
To determine the Kd of the enzyme, different concentrations of OH-AEP were mixed with anaerobic FeOF. The resulting Kobs for seven such experiments were then plotted against substrate concentration (Figure 26). The resulting plot was then fit with a hyperbolic equation to extract Kd, yielding $77.06 \pm 53.25 \, \mu M \, s^{-1}$. This value is on par with that obtained for PhnZ.  

![Figure 26. Determination of Kd by plotting Kobs versus substrate concentration.](image)

Finally, a single turnover assay was conducted qualitatively by mixing a stoichiometric amount of oxygenated buffer with substrate-saturated protein. By measuring absorbance across all wavelengths (Figure 27), it was seen that the enzyme went from completely substrate bound, to substrate-free, and then bound substrate again. These results show that the enzyme returns to the [II/III]-OH-AEP state after catalyzing substrate turnover, and is ready to react with another equivalent of substrate.
Figure 27. Stopped-flow absorption spectra showing a single turnover experiment in the HD-domain of the FeOf protein. Pictured are eight representative time traces collected from the incubation of 2 mM OH-AEP, 150 μM FeOf protein mixed with an equal amount of oxygenated buffer. (a) Panel A shows the relative absorbance collected from a photo-diode array at wavelengths 240-720 nm. (b) Panel B shows a difference spectrum with the change in absorbance versus wavelength constructed subtracting the spectrum at time zero from all eight representative traces.

Figure 28. Stopped-flow absorption spectrum at 397 nm showing a single turnover experiment in the HD-domain of the FeOf protein. The traces started at roughly zero (substrate bound), increased to respective maxima and minima by 0.1s (O₂ binding and catalytic activity), and decayed back to zero by the end of the assay (substrate bound), thus showing one enzyme turnover.
However, measurement of absorption at 397 nm showed that the concentration of substrate-bound protein did not quite return to pre-O$_2$ mixing levels. This could indicate that some of the active form of the enzyme may be oxidatively inactivated, remaining in the Fe$_2$[III/III] state (Figure 28). Further experiments will be able to more quantitatively assess the nature of the single turnover with the FeOf protein.

**Experimental**

**General**

*Spectroscopy:* Protein and DNA concentrations were determined in triplicate using a UV-Vis spectrometer (Cary 60), scanning at A$_{280}$ and A$_{260}$ respectively, with a background correction at 800 nm. The molar absorption coefficient of the FeOf protein is 83.8 mM$^{-1}$cm$^{-1}$. Acquisition of $^{31}$P NMR spectra was carried out on a Varian 400 MHz spectrometer. Chemical shifts are reported in ppm, referenced to a sample of H$_3$PO$_4$.

*Degassed protein samples (FeOf as isolated, FeOf S200 purified, PhnY, PhnZ):* Protein (2 mL) was thawed on ice and transferred to a pre-cooled 20 mL Schlenk flask on ice equipped with a stir bar. The Schlenk flask was sealed with a septum and a zip tie and attached to a standard Schlenk line equipped with argon gas and vacuum. The headspace in the flask was exchanged with 10 evacuation and refill cycles and sealed under argon gas.

*Glovebox materials and experiments:* all anaerobic experiments were carried out under a nitrogen/hydrogen atmosphere (~2% hydrogen) in an anaerobic chamber (Coy). All materials used in our experiments were rendered O$_2$-free. Buffers were made O$_2$-free using a Schlenk line equipped with argon gas and vacuum, by employing four evacuation and refill cycles (20 minutes
and 10-15 minutes respectively). Buffers were allowed to equilibrate overnight in the glovebox prior to the experiments.

Sterile technique: all experiments involving live cultures of *Eschericia (E) coli* cells were carried out using sterile techniques. All disposable materials (e.g. pipette tips, burettes, 1.5 mL Eppendorf tubes, etc.) were sterilized by autoclaving prior to use. Additionally, all Luria-Bertani (LB) media were sterilized by autoclaving.

1. Protein expression and purification

1.1. Transformation

*Fonsaecae (F) multimorphosa* *feOf* plasmid was obtained using a Monarch™ Plasmid Miniprep Kit (New England BioLabs®). Chemically competent Lemo BL21 (D3) *E. coli* cells (100 µL) were mixed with the FeOf plasmid (incubated on ice for 15 minutes, heat shocked for 90 seconds, and incubated on ice for a final 2 minutes). Cells (500 µL) were plated on LBK (Luria-Bertani broth with kanamycin) and incubated at 37 °C overnight to select for kanamycin antibiotic resistance.

1.2. Expression of FeOf protein

Preparation of standard LB media used Lennox granulated powder from Fischer BioReagents dissolved in de-ionized Milli-Q® water (20 g Lennox to 1 L water) and titrated to pH 7.3 using Tris buffer. Two colonies from the LBK plate were then inoculated in 150 mL of LB media with 50 µg / mL of Kanamycin overnight at 30°C with shaking at 220 rpm. Six 1 L flasks of LB (50 µg / mL) media were inoculated with 10 mL of the cell culture and incubated at 37 C, shaking at 220 rpm, for 2.5 hours. The cultures were heat shocked for 3 hours, and IPTG (250 mM), antifoam (2-3 drops), and Fe(NH₄)SO₄ (250 µM) was added. The cultures were incubated
overnight at 18°C with shaking (220 rpm) to express FeOf protein. Centrifugation at 4 °C, 7,000 rpm, for 15 minutes yielded pelleted cells (27.6 g), which were frozen in liquid N2 (27.6 g) and stored at -80 °C.

1.3. Lysis

The frozen cells were re-suspended by stirring in 150 mL of lysis buffer (50 mM MOPS, 300 mM NaCl, 10 mM imidazole) in a metal beaker covered with plastic wrap at 4°C. PMSF was added to the solution (150 μL of a 45 mg / mL prepared in ethanol). Ice was placed around the metal beaker, and the resulting suspension was sonicated for 45 minutes at 70% intensity (in cycles of 20-second pulses and 59 seconds of relaxation time). The lysed cells were centrifuged for 40 minutes, and the supernatant was taken off carefully to avoid any cell debris.

1.4. Purification

A nickel-NTA immobilized affinity chromatography column was prepared by equilibration with lysis buffer 50 mM MOPS, 300 mM NaCl, 10 mM imidazole, ~ 1 column volume) and kept at 4°C throughout the entire purification. The lysate was carefully loaded onto the column, after which a white-brown tinge was apparent on the column, suggesting bound protein. The column was washed with ~75 mL lysis buffer and ~75 mL wash buffer (50 mM MOPS, 300 mM NaCl, 20 mM imidazole). Protein was eluted using a high imidazole buffer (50 mM MOPS, 150 mM NaCl, 250 mM imidazole) in four fractions until elution from the column showed no protein using the Coomassie reagent. The four elution fractions were combined and concentrated using a Sorvall Legend XTR centrifuge in an Amicon® Ultra-4 centrifugal filter. The protein was re-buffered in a pH 6.5 buffer (50 mM MES, 10% glycerol) until the imidazole concentration was < 1 μM, producing about 7 mL of 807.9 μM FeOf protein with a purple tinge. The protein was separated
into 1 mL samples in Eppendorf tubes and stored at -80° C. The protein was further purified in small quantities by using an S200 gel filtration column, run with (50 mM MES, 0.2 mM βMe, 10% glycerol). Gel of purified protein shown in Figure 30.

1.5. Gel analysis of purification

SDS gels were prepared with a lower resolving gel (12% acrylamide, 0.1% SDS, 0.375 M Tris, pH 8.8) and an upper stacking gel (5.1% acrylamide, 0.1% SDS, 0.125 M Tris buffer, pH 6.8). Samples for running on the gel were prepared with 10 µL of the desired sample (W1, E1, etc.), 10 µL of 2X sample loading buffer, and denatured at 95°C for 5 minutes. The gel was run at 90V for 90 minutes, against a PageRuler protein ladder (prestained, purchased from ThermoFischer Scientific) and visualized using a Coomassie stain (Figure 29). Purified protein (MW 53952.7, determined using the ProtParam server- http://web.expasy.org/protparam/) was apparent in all four elution samples, with the greatest concentration in E2.

Figure 29. SDS-PAGE gel of FeOf protein, as isolated. Left to right: pellet, lysate, flow-through (FT), wash 1 (W1), wash 2 (W2), PageRuler protein ladder, elution 1 (E1), elution 2 (E2), elution 3 (E3), elution 4 (E4).
Figure 30. SDS-PAGE gel of FeOf protein purification using S200 column. Left to right: PageRuler protein ladder, sample of discarded fractions, isolated pure FeOf protein (10x dilution), FeOf protein as isolated before S200 column. This gel shows that the S200 purification removed higher-molecular weight protein aggregates.

1.6. Iron quantification

The FerroZine assay was used to estimate equivalents of iron per protein. Protein stock (150 µM) was combined with 10% trichloroacetic acid to form a total volume of 100 µL, denaturing the sample, and then spun for 2 minutes at 17,000 x g. The supernatant was taken, and ddH2O was added to a total volume of 500 µL. Ascorbic acid (20 µL, 75 mM), FerroZine (20 µL, 10 mM), and saturated NH4OAc (120 µL) were added, and the absorbance at 562 nm was measured. To chelate out the iron from the protein, 200 µL of as-isolated protein was mixed with 2 µL of 0.5 M EDTA (pH 8) for 1 hour, and then diluted 10x and re-buffered with pH 6.5 buffer (50 mM MES, 10% glycerol).

Table 1. Equivalents of iron per protein for native protein (as isolated and purified) and protein after chelation with EDTA.

<table>
<thead>
<tr>
<th>Protein form</th>
<th>Equivalents of iron per protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>As-isolated</td>
<td>2.04</td>
</tr>
<tr>
<td>After chelation with EDTA</td>
<td>1.28</td>
</tr>
<tr>
<td>S200 purified</td>
<td>1.46</td>
</tr>
</tbody>
</table>
2. Generation of *Fm* FeOf variants

2.1. Primers

Two *Fm* FeOf variants were generated to produce separate mutations that were either unable to coordinate the mono-iron site (α-KG dependent PhnY-like domain) or the diiron site (PhnZ-like HD domain). These two mutations, respectively H117 A and D335A, are shown in Table 2. Primers were synthesized by Integrated DNA Technologies (IDT).

<table>
<thead>
<tr>
<th></th>
<th>H117A</th>
<th>D335A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>GCG CCG <strong>CAC</strong> GTT GAT AGC</td>
<td>CTG CTG <strong>CAT</strong> GAC ATT GGT CAG</td>
</tr>
<tr>
<td>Forward</td>
<td>GCG CCG <strong>GCA</strong> GTT GAT AGC</td>
<td>CTG CTG CAT <strong>GCG</strong> ATT GGT CAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCG GGT AGC GTG GGC TTT</td>
<td>CAA ACC GTT GCG GCG GCG</td>
</tr>
<tr>
<td>Reverse Complement</td>
<td>AAA GCC ACC GCT ACC CGC</td>
<td>CGC CGC CGC AAC GGT TTG</td>
</tr>
</tbody>
</table>

2.2. Mutagenesis and Transformation

A BioLabs® Qs Mutagenesis Kit was used to prepare samples for PCR of the IDT primers. A BIO-RAID T100 Thermal Cycler was used for PCR with the following parameters: denaturation cycle (98 °C, 1 minute), 25 cycles of denaturation, annealing, and extension (respectively 98 °C, 10 seconds; 61.4-60.9 °C, 30 seconds; 72 °C, 3.5 minutes), and final extension (72 °C, 2 minutes). The product of the KLD reaction carried out using the same mutagenesis kit was incubated with 25 μL of NEB competent *E.Coli* cells on ice for 30 minutes. The cells were then heat shocked at 42 °C for 45 seconds, and incubated on ice for 5 minutes. SOC growth medium (500 μL) was
added, and the cells were incubated for 90 minutes at 37 °C. 300 μL of the culture was plated onto LBK selection plates and incubated overnight at 37 °C.

2.3. Sequencing

Small cultures of each mutant were made in duplicate by incubating a single colony in LB media (5 mL, with 50 μg/mL kanamycin) and were incubated at 37 °C overnight at 220 rpm. The cultures were pelleted into 1.5 mL Eppendorf tubes by centrifuging a series of 1.4 mL fractions at 11,000 x g for 1 minute. A Monarch™ Plasmid Miniprep Kit (New England BioLabs®) was used to obtain each plasmid. Sequence data was obtained from Genewiz showing that all four cultures had the correct mutant sequence.

2.3. Expression and purification of Feof variants

All further expression and purification steps were carried out as described for the purification of the wild type protein (section 1).

3. Thermal shift assays

A MicroAmp® Fast Optical 96-well plate was used for thermal shift assays conducted on an Applied Biosystems StepOnePlus Real-Time PCR machine. Melting curves were run 95°C using StepOne software 2.0. Samples were prepared to a total volume of 25 μL containing 10 μM protein, 10X SYPRO dye.

3.1. Buffer optimization for FeOf protein. A thermal shift assay was conducted on FeOf at pH’s 4, 5, 5.5, 6.5, 7, 7.5, 8, 9, 10, and 11 and at the same pH’s in the presence of (separately) salt and βMe. Selected data are shown below, indicating that the FeOf protein is relatively stable from pH 5.5 to pH 9.
3.2. Buffer optimization for PhnY protein. A thermal shift assay was conducted on PhnY protein at pH’s 4, 5, 5.5, 6.5, 7, 7.5, 8, 9, 10, and 11 in the presence of 10 μM FeNH₄SO₄ (1 equivalent). Samples were prepared anaerobically in the glovebox.

3.3. Buffer optimization for PhnZ protein. A thermal shift assay was conducted on PhnY protein at pH’s 4, 5, 5.5, 6.5, 7, 7.5, 8, 9, 10, and 11 in the presence of 8 μM FeNH₄SO₄ (0.8 equivalents). Samples were prepared anaerobically in the glovebox.

3.3. Effect of iron on the stability of the FeOf protein. A thermal shift assay was conducted on FeOf protein at iron concentrations varying from 0 to 5 equivalents of FeNH₄SO₄. The data collected indicated that there is no discernable trend in stability with increased concentration of iron. Samples were prepared anaerobically in the glovebox.

4. Activity Assays

Multiple turnover experiments using both the FeOf and PhnY/Z systems were conducted on a variety of substrates (2-AEP, 3-APP, TMAEP, and propyl-AEP), in a variety of different pH’s, and reactions were carried out for different times. Extent of substrate conversion was monitored by quenching the reaction and measuring phosphorous signal by NMR spectroscopy. Reported here is a general procedure for all experiments that were conducted in this fashion. For each experiment, a 500 μL sample was prepared anaerobically in the glovebox (see below) and then mixed with aerobic buffer outside the glovebox 500 μL to a total reaction volume of 1 mL.

Anaerobic protein (10 μM) was mixed with buffer in a small gas chromatography vial charged with a stir bar. FeNH₄SO₄ stock (50 μM) and sodium ascorbate (1 mM) were added simultaneously, and the mixture was allowed to stir briefly. Next, α-KG (3 mM), and 2-AEP (2 mM) were added sequentially. All samples were oxygenated while stirring via flushing the
headspace of the vial with air during the course of the reaction. Reactions were quenched with FeKCN$_6$ (25 μL of a 200 mM stock solution) and allowed to stand for two minutes. EDTA (25 μL, 0.5 M, pH 8) was added and samples were denatured at 80°C for 10-15 minutes. Spinning at 17,000 x g for 2 minutes removed the precipitate. Samples for NMR spectroscopy were prepared with 500 μL of the supernatant, 120 μL of D$_2$O, and 6 μL of EDTA (0.5 M, pH 8).

5. Generation of the different redox forms of Fs FeOf

Aerobically isolated FeOf predominantly contains the Fe$_2$(III/III) cofactor: preparation of reduced forms of the cofactor was accomplished in the following manner. The Fe$_2$(II/II) and Fe$_2$(II/III) states were generated by the anaerobic addition of 10-fold excess sodium dithionite or sodium ascorbate, respectively, followed by 30-60 minutes of incubation at room temperature. Proteins were then passed over a PD10 column pre-equilibrated with 50 mM MOPS (pH 6.5) to remove dithionite/ascorbate and their oxidized products from the protein. Protein aliquots were stored under liquid nitrogen until use. Whereas the fully reduced [Fe$_2$(II/II)] and fully oxidized [Fe$_2$(III/III)] cofactor forms can be generated to homogeneity, in contrast, the mixed-valent form of the cofactor in WT PhnZ can be accumulated typically to yields of ~55-70%.

6. Stop-flow Absorption (SF-Abs) spectroscopy

6.1. General

Stopped-flow absorption experiments were carried out on an Applied Photophysics SX-200 stopped-flow spectrometer located in an anaerobic chamber. Sample syringes were maintained at a temperature of 5 °C using a continuous-flow water jacket and reaction progress was monitored using a photodiode array detector. Two syringes were used for each experiment, one containing the substrate (either OH-AEP or O$_2$), and one containing 150 μM of FeOf Fe$_2$(II/III). Stopped-
flow absorption data were collected by rapidly mixing the enzyme with OH-AEP or dioxygen. Each drive of the syringes introduced 60 µL of the sample in each syringe to the IR cell: note therefore that the reaction mixture contained concentrations of substrate and protein that were half of that loaded in the syringes.

6.2. Transient kinetics experiments

Absorption data was collected measuring binding of OH-AEP to the diiron site of FeOf over time. As OH-AEP binds to the Fe(II/III) state, a shift towards 400 nm is observed, indicating binding of substrate, and a corresponding decrease at 490 nm, indicating a decrease in the substrate-free mixed-valent state. This reaction was measured for seven concentrations of OH-AEP: 50 µM, 100 µM, 200 µM, 240 µM, 500 µM, 1000 µM, 2000 µM. The resultant traces were fit to an exponential growth or decay to extract the rate of binding (k_{obs}) for a given substrate concentration. A plot of substrate concentration versus k_{obs} was then fit with a hyperbolic equation, and the initial slope extracted to determine the second order rate constant (K_1k_2).

6.3. Single turnover experiment

A single assay was carried out mixing oxygenated buffer with substrate saturated enzyme (> 2 mM OH-AEP). Oxygenated buffer was brought into the glovebox in an air tight vial and loaded into a sample syringe. The oxygenated buffer was mixed with the enzyme for 60 seconds and an absorption spectra was collected, allowing qualitative measurement of a single turnover. With more experiments, binding of OH-AEP to FeOf and reaction with dioxygen could be fit according to a rapid-equilibrium, two-step process according to the following general equation: E+S ⇌ E•S ⇌ E•X. However, given the limited data, a quantitative assessment of single turnovers is not yet complete.
7. Mössbauer spectroscopy

M9 minimal media (1 L) was prepared with glucose (0.6%), FeNH₄SO₄ (250 μM), CaCl₂ (100 mM), MgSO₄ (2 mM), and kanamycin (50 μg/mL). A pre-culture with 100 mL of M9 media and two colonies of FeOf wild-type was grown overnight at 30°C, 220 rpm. A larger scale (1 L) culture of M9 media was inoculated with 25 mL of the pre-culture, and incubated at 37°C, 220 rpm, for 4 hours. Lysis and purification were carried out as explained in section 1, with the modification that during protein expression Fe⁵⁷NH₄SO₄ was added in place of regular FeNH₄SO₄ during induction.

Figure 31. SDS-PAGE gel of Fe⁵⁷Of protein, as isolated. Left to right: pellet, lysate, flow-through (FT), wash 1 (W1), wash 2 (W2), elution 1 (E1), elution 2 (E2), elution 3 (E3), elution 4 (E4). PageRuler protein ladder.

After verifying protein purity by using SDS-PAGE, the purified protein was concentrated down to 250 μL and were transferred aerobically in a Mössbauer cup and the frozen in liquid nitrogen.
Mössbauer spectra were recorded on a spectrometer from WEB Research, equipped with a Janis SVT-400 variable-temperature cryostat. The external magnetic field (78 mT) was parallel to the γ-beam. All isomer shifts are quoted relative to the centroid of the spectrum of α-Fe at room temperature. Simulation of the Mössbauer spectra was conducted in the WMOSS spectral analysis package.

Conclusions

We have identified and characterized the third reported HD diirion oxygenase, isolated from the fungus Fonsecaea multimorphosa. Structural analyses and sequence alignments indicated that the protein contained both a PhnY-like (an α-KG dependent mono-iron oxygenase) and a PhnZ-like (HD-domain diiron oxygenase) domain. Expression and purification of this protein from E. coli cells yielded the native protein with 2.1 equivalents of iron per protein. Using Mössbauer spectroscopy, we determined that the iron in the native protein is almost exclusively found at the HD-diiron site. We carried out a preliminary substrate screening with a variety of naturally occurring and synthetic organophosphonates using $^{31}$P NMR. The fusion enzyme reconstituted with iron and α-KG catalyzes complete conversion of 2-aminoethylphosphonate to inorganic phosphate, with 200 turnovers in 140 minutes. This proved that the fusion enzyme does indeed show activity similar to that of the PhnY/Z system, presumably first hydroxylating 2-AEP to OH-AEP and then oxidatively cleaving the CP bond. Although the fusion enzyme can take a wider range of substrates that the PhnY/Z system, its natural substrate, like PhnY/Z, is 2-AEP.

Incubation of 3-aminopropylphosphonate with the fusion enzyme resulted in complete conversion to phosphate, with 200 turnovers of substrate in 140 minutes, whereas the combined PhnY/Z system afforded only ~72 turnovers in the same amount of time. This observation suggests that there is a tight coupling in the fusion system superior to that of the individual enzymes, perhaps
endowing the fusion protein with an extended substrate selectivity. Furthermore, thermal shift stability analysis showed that the fusion protein folds in roughly two components, with both putative PhnY-like and PhnZ-like domains showing greater thermal stability than of native PhnY or PhnZ.

PhnY/Z comprise a relatively new pathway for the metabolic cleavage of the C-P bond in AEP, one of the most abundant organophosphonates in nature.\textsuperscript{3-4} We have shown here that the fusion protein obtained from \textit{F. multimorphosa} is capable of the same type of cleavage. This characterization of a novel fusion protein further extends the understanding of the HD-domain, investigating the third known HD-domain oxygenase. Furthermore, it provides some hints as to the rationale behind coupling two enzymes together in a fusion protein: although the fusion protein seems to be at a loss for brute conversion speed, it gains in stability and substrate scope.

**References**