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A family of structural and functional models for the active site of a unique dioxygenase: Acireductone Dioxygenase (ARD)

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Abstract

We report the synthesis and biomimetic activity of a family of model complexes with relevance to acireductone dioxygenase (ARD), an enzyme that displays dual function based on metal identity found in the methionine salvage pathway (MSP). Three complexes with related structural motifs were synthesized and characterized derived from phenolate, and pyridine N₄O Schiff-base ligands. They display pseudo-octahedral Ni(II)-N₄O ligand coordination with water at the sixth site, in close alignment to the structure in the resting state of ARD. The three featured complexes exhibit carbon-carbon bond cleavage activation of lithium acetylacetonate, which was used as a model enzyme substrate. Computationally derived mechanistic routes for the observed reactivity consistent with experimental conditions are herein proposed. The mechanism suggests the possibility of Ni(II)-substrate interactions, followed by oxygen insertion. These results constitute only the third functional model system of ARD, in an attempt to further advance biomimetic contributions to the ongoing debate of ARD's unique metal mediated, regioselective oxidative cleavage.

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Supporting information:

X-ray data collection and refinement details, UV-Visible spectra, ¹H NMR characterization and reactivity spectra, elemental analysis, and ESI-mass spectral data.

Accession codes:

CCDC: 1577098, 1985088 and 1985383 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Synopsis of Graphical Abstract

A summary of the methionine salvage pathway and acireductone dioxygenase's (ARD) role in this context. One of the biomimetic complexes from this work is displayed alongside a skeletal cartoon of the active site of ARD. This figure highlights the connection between the enzyme and our model system.

1. Introduction:

In this article, a novel set of structural and functional biomimetic models for the enzyme nickel acireductone dioxygenase (Ni-ARD) is introduced. These complexes represent only the third example of a reactive biomimetic model for the enzyme, and the first that will permit the systematic exploration of the role that the electronic environment of the metal plays in function, mechanism, and ultimately its role in promoting enzymatically derived disease pathways for Ni-ARD. This work thus contributes to the very relevant and ongoing debate concerning the role and mechanism of action that ARD has in disease.

Acireductone Dioxygenase (ARD) plays a key role in the ubiquitous methionine salvage pathway (MSP) in animals, plants, and bacteria.¹ The MSP uses methylthioadenosine (MTA) derived from S-adenosyl methionine (SAM) after polyamine synthesis in animals and ethylene in plants. MTA is an inhibitor of polyamine synthesis and transmethylation reactions.² It is known that the inhibition of MTA halts DNA replication and elevated amounts of polyamines are associated with tumor formation.³ ARD catalyzes the penultimate step in MSP, the oxidative cleavage of the substrate *acireductone* (1,2-dihydroxo-3-keto-5-(thiomethyl)pent-1-ene) into 2-keto-4-(thiomethyl) butyrate (KMBT-methionine precursor) and formic acid. This reaction occurs when iron is bound to the active site of the enzyme and is known as the "on-pathway" route. In contrast, when nickel is bound to the active site, an "off-pathway" shunt leads to the formation of 3-

(methylthio)propionate (MTP), formate, and carbon monoxide (Figure 1). This difference in the regioselective oxidation of substrate can be ascribed to the identity of the metal ion. ARD is the only known example of a metalloenzyme displaying such behavior. Recently, a mouse analogue of ARD (MmARD) was structurally characterized at high resolution,⁴ and a human analogue of ARD (HsARD) is now available at low resolution.⁵ Both of these mammalian analogues showed via *in vitro* studies to perform the same metal dependent chemistry as that observed in the bacterial ARD.^{4,6,7} Furthermore, metal promiscuity binding studies showed that MmARD and HsARD display identical “*off-pathway*” chemistry when bound to Co^{2+} or Mn^{2+} .^{6,7} It has been hypothesized that the “*off-pathway*” function of ARD, which is observed in the presence of metals relevant in mammals (cobalt, iron and manganese), could lead to cells having anti-apoptotic behavior in cancerous cells mediated by the production of CO, a known anti-apoptotic signaling molecule,^{8,9} and neurotransmitter in mammals.¹⁰ The oxidative cleavage of acireductone substrate is generally accepted to follow a Lewis acid mediated activation followed by oxygen insertion, and not a more traditional path of oxygen activation promoted by other dioxygenases.¹¹

In the resting state of the enzyme, the metal ion in ARD is coordinated to the protein backbone in a pseudo-octahedral environment, by three histidine and one glutamate residues, with the remaining coordination sites occupied by two water molecules (Figure 2). The structure of the active site is conserved regardless of its enzymatic context.¹

The actual mechanism for activation of acireductone is currently under debate among four working hypotheses.^{7,12,13,14} *The first*, deemed the *chelate hypothesis*, proposes that the regioselectivity of the reaction, and hence the differences of product formation, result from the mode of substrate binding to different metals, Fe vs. Ni. This difference in substrate coordination promotes a different oxygenation pathway by encouraging only certain intermediates to be accessible.¹² A *second working mechanistic proposal*, challenging the chelate hypothesis comes from recent computational work. These studies suggest that differences in the profile of oxygenation product formation are influenced by the charge transfer differences innate to Fe and Ni. Where iron is more capable of donating electron density to the substrate creating a pseudo-redox state that is simply not possible with nickel.¹³ A *third hypothesis* is derived from biomimetic modeling work.¹⁴ These studies suggest that the regioselective behavior of ARD is modulated by the access of water to the active site of the enzyme. The models’ biomimetic reactivity appeared to be susceptible to the presence or absence of water leading to off-pathway type products only when water was rigorously removed regardless of the type of metal used. In this work it was shown that coordination mode of substrate did not affect regioselectivity, which is in contrast to the chelate hypothesis. A *fourth working hypothesis* was recently deduced from computational and experimental work done on human ARD (HsARD).⁷ These studies suggest that human ARD goes through an oxygen activation step, where oxygen is bound to the active site followed by substrate oxidation in the presence of human relevant metals Fe^{2+} and Co^{2+} . This is in direct contrast with earlier studies indicating no evidence for redox activity of the metal at the active site.¹⁵

Taken together this body of work highlights the need for further clarification on how the enzyme mediates the proposed regioselective oxidation, and more importantly the need for

more and improved active site models of ARD. This is relevant because of ARD's possible involvement in mammalian systems with the anti-apoptotic behavior in cancerous cells mediated by the production of CO. Those studying ARD agree that understanding the mechanistically driven regioselectivity on these enzymatic reactions is essential to clarifying ARD's "off-pathway" chemistry, its involvement in disease, and other moonlighting functions.^{1, 4, 15, 16, 17}

To our knowledge, there are only two functional biomimetic models for ARD. The Ni(II) complex [(6-Ph₂TPA)Ni(CH₃CN)₂](ClO₄)₂ uses the N4 chelating ligand 6-Ph₂TPA (N,N-bis[(6-phenyl-2-pyridyl)methyl]-N-(2-pyridylmethyl)amine). Extensive biomimetic reactivity studies have been reported for this model using acireductone substrate analogues.^{14,17,18,19} This work contributed observations aimed at clarifying the ongoing mechanistic uncertainty, specifically challenging the chelate hypothesis¹⁴ mentioned above. Recent work with macrocyclic N4 tetradentate ligands (*N,N'*-di-benzyl-2,11-diaza[3,3](2,6)pyridinophane), and their corresponding Ni(II) complexes, reported the oxidative cleavage of an ARD substrate analogue under atmospheric oxygen and ambient conditions.²⁰ Although these are functional models, the N4 systems lack all of the structural and electronic characteristics to accurately mimic the electronic environment of ARD's active site, specifically the introduction of the oxygen moiety corresponding to the glutamate donor in ARD. It is important to note that additional work reported the use of an N3 chelating ligand (2,6-bis(1-methylbenzimidazolyl)pyridine) to make Ni(II) complexes that were shown to be reactive with substituted diketones towards C-C oxidative bond cleavage analogous to the enzymatic reactivity.²¹ These results, however, were recently put into question suggesting that activation of electron deficient diketone substrates was not reproducible in these complexes.²² The biomimetic precedents argue in favor of models that more closely align with the electronic and structural characteristics of the enzyme. In the present work we introduce the synthesis and characterization of a family of structural models closely related to ARD, which more closely mimic the electronic structure of the active site and constitute proof of principle for future systematic structure-function studies of ARD's oxidative reactivity using model complexes. We report here preliminary results of their biomimetic carbon-carbon cleavage reactivity towards a substrate mimic, and a proposed mechanism for the observed reactivity. This work expands ongoing biomimetic studies to advance the aforementioned debate regarding the unique reactivity of ARD.

2. Experimental section

2.1 Materials and methods

The preparation and manipulation of air-sensitive compounds were performed using standard Schlenk techniques under an N₂ atmosphere. Reagents and solvents were purchased from commercial suppliers of the highest available purity and used without further purification unless otherwise noted. Solvents (MeOH, MeCN, 2-Butanone, and Et₂O) were dried using 4 Å molecular sieves. Solvents were degassed via freeze-pump-thaw cycles.

2.2 Physical methods

¹H NMR spectra were recorded on a Varian 300 MHz spectrometer at room temperature and referenced to a residual deuterated solvent. UV-vis spectra were recorded in a 1 cm quartz cuvette on a Varian Cary 100 Bio spectrophotometer. ATR-FTIR spectra were recorded with an Agilent/Cary 630 FTIR KBr or ZnSe engine. The electrochemical data were obtained using CH Instrument 600E Electrochemical workstation. ESI-Mass spectral data were collected using Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS equipped with a Jet Stream electrospray ionization (ESI) source. Elemental analyses were performed by Galbraith Atlantic Microlabs, Norcross, GA.

2.3 Synthesis of ligand precursors 6-H-DPEN (LP1) and 6-Me-DPEN (LP2):

The ligand precursors N,N-Bis(2-pyridylmethyl)ethane-1,2-diamine (**6-H-DPEN**, **L1**) and N,N-Bis(6-Me-2-pyridylmethyl)ethane-1,2-diamine (**6-Me-DPEN**, **L2**) were synthesized using slight modifications to previously reported synthetic procedures.^{23–25} *N*-acetylenediamine (141 μ L, 1.03 mmol), and the corresponding aldehyde (2-pyridinecarboxaldehyde or 6-Me-2-pyridinecarboxaldehyde, 139 μ L, 1.03 mmol) were dissolved in 10 mL of 1,2-dichloroethane and allowed to stir under an N₂ atmosphere at room temperature. The reaction progress was monitored via thin layer chromatography using ninhydrin stain to screen for any unreacted amine. After ~30 minutes, an equivalent of NaBH(OAc)₃ (0.4375 g, 2.89 mmol) was added to the reaction mixture. Continual monitoring via TLC was done as the mixture was allowed to react for 2 hours. After this period an additional equivalent of the corresponding aldehyde and NaBH(OAc)₃ were added to the reaction mixture and allowed to react for an additional 30 minutes. The reaction was then quenched with 15 mL of 2 M NaOH and extracted with dichloromethane and washed with saturated sodium chloride solution, followed by drying over anhydrous sodium sulfate. The solvent was removed under reduced pressure, then further dried under vacuum, resulting in a brown oil in 88% yield. The acetyl protecting group was removed by adding 20 mL of 5 M aqueous hydrochloric acid and allowed to reflux for 24 hours under an N₂ atmosphere. The resulting solution was treated with careful addition of 5 M NaOH and the organics were extracted with dichloromethane. Afterwards, the organics were dried over anhydrous KOH and dried under reduced pressure. Spectroscopic details of ligand precursors can be found in the supporting information document.

2.4 Synthesis of metal complexes

2.4.1 [Ni^{II}(O^{Ph}N₄(6-H-DPEN)(H₂O)](OTf) (1): The synthesis and characterization of complex **1** was published in earlier work.²³

2.4.2 [Ni^{II}(O^{Ph}NO₂N₄(6-Me-DPEN)(H₂O)](OTf) (2): Synthesis of the metal complex **2** was formed via an in-situ reductive amination reaction. The 6-Me-DPEN ligand precursor (192 mg, 0.751 mmol) was dissolved in 5 mL dichloroethane with 4 Å molecular sieves. One equivalent of the corresponding aldehyde, 2-hydroxy-5-nitrobenzaldehyde (126 mg, 0.751 mmol) was added and the mixture was allowed to react for 3 hours. The solvent was then removed under reduced pressure and further dried under vacuum. 5 mL of MeOH and one equivalent of N,N-diisopropylethylamine (0.0759 mL, 0.751 mmol) were added and allowed

to stir for 20 minutes. Nickel trifluoromethanesulfonate (268 mg, 0.751 mmol) was then added and the reaction was allowed to stir under dynamic nitrogen at room temperature for two days. The resulting solution was filtered through a frit under vacuum and then pumped down under reduced pressure. The resulting oil was washed with diethyl ether three times and then pumped down once again. A green powder was isolated.

Yield= 74.2% (351.7 mg, 0.557 mmol). Electronic absorption spectrum (MeOH): λ (nm) (ϵ ($M^{-1} cm^{-1}$)): for complex **2**: 235 (5128), 371 (3896), 575 (99). IR: C=N: 1654 cm^{-1} . We were not able to isolate complex **2** as an analytically pure sample. HRMS (ESI-MS, m/z): calcd. for $[C_{23}H_{24}N_5NiO_3]^+$: found: 476.1226, calculated: 476.1227.

2.4.3 $[Ni^{II}(O^{PhNO_2}N_4(6-H-DPEN)(H_2O)](OTf)$ (3**):** The synthesis of complex **3** followed the procedure outlined for complex **2** with the alteration of using the 6-H-DPEN ligand precursor (63.5 mg, 0.262 mmol). A yellow powder was isolated. Yield= 80% (129.1 mg, 0.2096 mmol). Electronic absorption spectrum (MeOH): λ (nm) (ϵ ($M^{-1} cm^{-1}$)): for complex **3**: 237 (9386), 381 (6879), 567 (115). IR: C=N: 1650 cm^{-1} . Elemental Analysis for $NiO_7N_5C_{22}H_{22}SF_3$ Calcd: C, 42.88%; H, 3.60%; N, 11.37%. Found: C, 43.14%; H, 3.49%; N, 11.34%. HRMS (ESI-MS, m/z): calcd. for $[C_{21}H_{20}N_5NiO_3]^+$: found: 448.09170, calculated: 448.0914.

2.5 X-ray Crystallography:

Single crystals of **1–3** were used for X-ray crystallographic studies. This analysis was performed at the University of Texas at Austin X-ray facility laboratories. X-ray data collection was done on a Rigaku AFC12 diffractometer with a Saturn 724+ CCD using a graphite monochromator with MoK α radiation ($\lambda = 0.71073 \text{ \AA}$). The data were collected at 100 K using a Rigaku XStream Cryostream low temperature device. Crystal data, refinement parameters, and additional details can be found in the supporting information for this manuscript.

2.6 Oxidative reactivity studies:

In a typical experiment, to a Schlenk reaction flask were added ~20 mg of the appropriate nickel complex (~0.035 mmol), 5 mol equivalents of lithium acetylacetonate, and these were dissolved in 1 ml CD_3OD and heated to 35 °C under a dynamic atmosphere of oxygen gas. This was done by flowing oxygen gas via the Schlenk line onto the reaction flask. The reactions were allowed to stir for at least 24 hrs. with ongoing in-situ monitoring thereafter via 1H -NMR by removing the reaction mixture and directly inserting into the NMR tube.

2.7 Computational methods:

Computational studies employed the Gaussian 09 software package. For geometry optimizations - and to obtain enthalpic and free energy corrections - the B3LYP functional was utilized in conjunction with the SBKJC pseudopotential/valence basis set for nickel while the 6-31+G(d) all-electron basis set was used for main group elements. At the optimized geometries, single point energy calculations with a larger basis set on the main group elements, 6-311++G(d,p), were conducted along with solvent (acetonitrile and methanol were modeled within the SMD continuum solvation model) and dispersion (GD3-

BJ) corrections. Intrinsic reaction coordinate calculations were conducted to confirm all transition states. All reported energetics are in kcal/mol and assume standard temperature and pressure.

3. Results and Discussion

3.1 Synthesis and characterization of metal complexes $[\text{Ni}^{\text{II}}(\text{O}^{\text{Ph}}\text{N}_4(6\text{-H-DPEN})(\text{H}_2\text{O}))(\text{OTf})]$ (**1**), $[\text{Ni}^{\text{II}}(\text{O}^{\text{PhNO}_2}\text{N}_4(6\text{-Me-DPEN})(\text{H}_2\text{O}))(\text{OTf})]$ (**2**), and $[\text{Ni}^{\text{II}}(\text{O}^{\text{PhNO}_2}\text{N}_4(6\text{-H-DPEN})(\text{H}_2\text{O}))(\text{OTf})]$ (**3**).

This work builds from our previously published structural model of ARD (**1**),²³ and takes advantage of its modular ligand design that permits the synthesis of a family of structurally related compounds. Complexes **2** and **3** are structural derivatives of **1** and were synthesized via the in-situ condensation of ligand precursor $[\text{N}',\text{N}'\text{-bis}(2\text{-pyridylmethyl})\text{ethane-1,2-diamine}; 6\text{-H-DPEN (LPI)}$ and $[\text{N}',\text{N}'\text{-bis}(6\text{-methyl-2-pyridylmethyl})\text{ethane-1,2-diamine}; 6\text{-Me-DPEN (LP2)}$, with the corresponding aldehyde (salicylaldehyde **1**, and 2-hydroxy-4-nitrobenzaldehyde **2,3** in the presence of N,N -diisopropylethylamine as an auxiliary base (Scheme 1). The ligand precursors LPI , and LP2 were synthesized in a two-step reductive amination via slight modification of existing synthetic procedures (see Supporting Information).^{23–25} Given that ARD's oxidative cleavage of substrate is proposed to occur via Lewis acid mediated substrate activation, complexes **1 – 3** aim to modulate the metal's Lewis acidity via structural modifications. Specifically complexes **2** and **3** introduce a 4-nitro substituent to the phenolate donor moiety, and **2** substitutes an additional 6-Me group on the pyridine donors of the ligand (Scheme 1). Recent work has demonstrated the importance of analogous modulation of the ligand's sterics and Lewis acidity plays on reactivity of model complexes.^{25,26} The metal complexes in this work aim at addressing questions about the specific role that Lewis acidity might play in the observed on- and off-pathway functions of ARD.

The resulting novel N_4O nickel complexes were isolated as yellow-green crystals from slow vapor diffusion crystallizations of diethyl ether into 2-butanone solutions of the corresponding complex. The molecular structures of complexes **1**, **2**, and **3** were obtained via X-ray diffraction and are shown in Figure 3.

The primary coordination sphere around the Ni center of each of the three complexes is a Schiff-base, N_4O donor framework with a water molecule occupying the sixth coordination site. The introduction of an O-donor moiety in these complexes is the first attempt at modeling the contribution of the glutamate oxygen of the ARD active site. *Furthermore, these complexes constitute the first examples of water-bound model systems emulating the resting state of the enzyme.* Key structural parameter comparisons across the complexes and with the active site of ARD are summarized in Table 1. Overall the mean Ni-N bond lengths are only slightly shorter for the model complexes (0.02 Å difference) when compared to the enzyme's average Ni-N bonds.⁴ The mean Ni-O^{Ph} is 0.08 Å shorter than the Ni-O_{Glu} bond length, as expected given donor basicity differences between a glutamate O-donor vs. a phenolate O-donor. The Ni-OH₂ average distance in the enzyme is only 0.03 Å longer than the mean Ni-OH₂ of the model compounds; this particular site is the one proposed to be labile in order to permit substrate binding. In spite of differences in coordination between the enzyme's active site and these structural models, given that these complexes carry an

additional nitrogen donor moiety (N₄O vs N₃O, Figure 2), we propose the incorporation of an oxygen donor, and the unique feature of water binding in complexes **1** – **3** provide sufficient structural similarities to ARD to justify these as structural models of ARD's active site. No biomimetic ARD activity has been reported for N₃O or N₄O chelating ligands.

When evaluating structural differences between the complexes as a result of the introduction of nitro groups or methylated-pyridyl derivatives, these modifications don't appear to have minor effects on the overall structures. It was hypothesized that nitro and methylated derivatives will lead to weaker metal-ligand interactions due to electron withdrawing and steric effects respectively. These modifications were introduced to impact the Lewis acidity at the metal center, and therefore indirectly affect eventual metal substrate interactions. Experiment shows the non-substituted derivative **1** has the shortest Ni-OH₂ bond length (0.05 Å and 0.06 Å shorter as compared to **2** and **3** respectively). Complex **2**, containing 6-methyl-pyridyl donors, shows the largest Ni-N_{py} bond lengths consistent with a steric effect imparted by the methyl substituents. Finally, the bonded water appears to prefer a trans N_{Imine} coordination in complex **1**, and trans N_{py} coordination in complexes **2** and **3**. Whether this is simply an artifact of the structural characterization thermodynamic preference, or if this position for the labile site remains in solution is yet to be determined. Density functional theory (DFT) simulations indicate, however, that the differences in free energies between the trans-N_{py} and trans-N_{imine} coordination isomers is very small, *ca.* 1 kcal/mol or less, suggesting that the particular isomer observed in the solid-state is likely quite sensitive to the crystal environment.

Cyclic voltammetry was performed on all three complexes (Supporting information Figures S4, S7 and S10). These display cathodic redox potentials for the presumed Ni(II) to Ni(III) redox couple. Complex **1** (unsubstituted), displays an irreversible wave with the most anodically shifted potential at 510 mV (vs. vs. Ferrocene-Fc/Ferrocenium-Fc+). The redox potential of **2** is cathodically shifted and displays a quasi-reversible wave with an E_{1/2} of 826mV (vs. Fc/Fc+). Complex **3** falls in between with a positive reduction potential E_{1/2} of 707 mV (vs. Fc/Fc+) and is also quasi-reversible. These reduction potentials are consistent with our structural observations and ligand design goals aimed at modulating the Lewis acidity of the complexes. Unsubstituted **1** is more thermodynamically able to access the Ni(III) state in comparison to the complexes containing sterically hindered and electron withdrawing modifications (**2,3**). The substitutions in **2** and **3** should increase the Lewis acidity of the metal by removing electron density at the metal center or preventing the approach of the pyridine donors as evidenced in the crystal structure of **2**. It is important to note that no other reduction/oxidation waves were observed suggesting that there is no redox-activity for the phenolic ligand framework.

The UV-Visible spectra of **1**, **2**, and **3** is available in the supporting information. All three complexes display charge transfer bands at approximately 375 nm (~18,000 M⁻¹cm⁻¹) and 290 nm (~4000 M⁻¹cm⁻¹), and weak d-d visible band at ~575 nm (~45 M⁻¹cm⁻¹). Based on literature examples, the band around 375 nm can be assigned to a ligand to metal charge transfer transition primarily associated with the phenolate ligand.^{19,27} The weak d-d bands are characteristic of hexacoordinated nickel(II) complexes. These are not solvent dependent,

thus suggesting that the hexacoordinate structure of the complexes remains intact in solution.

The ^1H -NMR spectra of complexes **1**, **2**, and **3** all display paramagnetically shifted signals consistent with features corresponding to penta- and hexa-coordinated Ni(II) complexes (Supporting information Figures S3, S5, S9).²⁸

3.2 Biomimetic reactivity observations of model complexes

In previous work, it was observed that complex **1** activated the crystallization solvent 2-butanone in the presence of oxygen giving rise to the formation of acetic acid, a probable carbon-carbon bond cleavage product.²³ This led to a systematic study to test the biomimetic oxidative activity of complex **1**, by probing its reactivity in the presence of oxygen towards a proof of principle model substrate, lithium acetylacetonate (LiACAC). This substrate serves as an analogue to ARD's native substrate 1,2-dihydroxy-3-keto-5-methylthiopent-1-ene (acireductone). Although LiACAC's symmetry does not allow for testing the regioselective chemistry observed in ARD, it has been used in the study of analogous model systems as a model compound for testing oxidative reactivity.^{20–22} Furthermore, this substrate is biologically relevant to other related oxygenases specifically acetylacetone dioxygenase (Dke1).²⁹

In a typical oxygenation reaction, a 0.035 M solution of the nickel complex in deuterated methanol was mixed in a 1:5 ratio with LiACAC in a dynamic atmosphere of oxygen and heated to 35°C while monitored for changes via ^1H -NMR of the reaction mixture. The ^1H -NMR spectra for the reaction mixtures between **1** and LiACAC under oxygenation and anaerobic conditions are shown in Figure 4 (top and bottom respectively). Under dynamic O_2 , the decrease of the peak corresponding to substrate LiACAC was observed, as measured by comparison to an internal standard, concomitant with the appearance of several signals in the region between 1–9 ppm of the spectrum (Fig 4 top). This is in direct contrast with the anaerobic reaction (Fig 4 bottom) which retains the relative ratio of internal standard to LiACAC and lacks specifically the features at 1.37, 1.91 ppm; the diamagnetic signals between 6.6 and 7.7 ppm; and the sharp singlet at 9.88 ppm. These results highlight the contrast between the reaction in the presence and absence of oxygen suggesting possible oxygen involvement. A control experiment under identical conditions of LiACAC under an O_2 atmosphere, in the absence of metal, resulted in the isolation of starting material LiACAC and no evidence of the presence of analogous peaks to those observed in the metal oxygenated reaction. Similarly, O_2 dependent reactivity was observed for complexes **2** and **3** where N_2 and O_2 reactions display different sets of peaks growing although slight differences between these reactions and those observed in reactions with **1** warrant further studies and will be reported in a future communication.

3.3 Proposed mechanism for reactivity

To account for these experimental observations indicating an oxygen dependence of the reaction we propose an oxygen mediated carbon-carbon bond cleavage of the acetylacetonate substrate (Scheme 2). The first step could correspond to the binding of acetylacetonate to the nickel complex with displacement of water. A binding event is

supported by NMR titration experiments that indicate changes in the paramagnetic spectra of the nickel complexes upon addition of 1.25 eq. of LiACAC substrate (Figure S11). Although, in ARD substrate binding of acireductone for Ni-ARD is proposed to be bidentate, at this time there is no structural enzymatic data to confirm that indeed the substrate in ARD is a bidentate chelate.¹ Furthermore when human relevant metals are bound to ARD, Fe, and Co, monodentate binding is the proposed binding mode of substrate.⁷ Computational (*vide infra*) and experimental observations (NMR diamagnetic aromatic signals Fig. 4) do not rule out a bidentate binding mode of substrate if one of the pyridine arms were to dissociate.

The second step in the mechanism is proposed as the dioxygen addition to the enolate bound species to form a peroxo type intermediate. This intermediate could then attack the carbonyl bound to nickel to give a second intermediate, a 1,2-dioxetane, which eventually collapses with the production of pyruvaldehyde and an acetate bound nickel complex (Scheme 2). Pyruvaldehyde has been shown to decompose via a variety of pathways, under the given experimental conditions, including the decomposition to CO₂ and acetic acid, as well as hydration oligomeric products.³⁰

Several observations provide support for the hypothesized reaction mechanism. Evolution of bubbles was observed in the aerobic reactions, which was more evident after the first 24 hours of reaction. To test for the possibility of this gas evolution corresponding to CO₂, the headspace of the reaction flask was purged into a vessel containing a saturated solution of Ca(OH)₂, which will react with CO₂ to produce CaCO₃ as a precipitate. After purging the headspace of the flask into the Ca(OH)₂ solution, small amounts of a milky white precipitate resulted. After ATR-FTIR analysis of the powder its identity was confirmed as CaCO₃ when compared to a control (Figure S12). To reiterate, CO₂ production supports the formation of pyruvaldehyde, a product exclusive to oxygenation of the substrate.

Additionally, we propose that the peak at 1.91 ppm observed in the NMR spectrum of the aerobic reactions with **1** (Figure 4 top) can be tentatively assigned to the -CH₃ of the acetate bound nickel(II) complex, another product of the oxygenation of LiACAC. We propose this based on comparisons of this peak to a peak observed in the independent synthesis of an acetate bound nickel(II) complex of **1** (Figure S13). The additional signals that grown during the oxidative reactivity could not be definitively assigned although we hypothesize, they might correspond to pyruvaldehyde hydration/solvation/oligomeric type products.³⁰

Finally, in most of the oxygenation reactions the formation of a small amount of an off-white cream-colored precipitate was observed as the reaction proceeded. The powder was isolated and analyzed via ATR-FTIR. The IR spectrum of the precipitate is shown in Figure S14. The spectrum displays strong bands at 950 cm⁻¹, consistent with C-O-C stretching, a band at 1379 cm⁻¹, and a broad peak at 3332 cm⁻¹ corresponding to an O-H stretch, all of these expected to be present with pyruvaldehyde hydrolytic type oligomers. This reactivity of pyruvaldehyde is well known in the literature.^{30a,e,f}

To further test the feasibility of the proposed mechanism density functional theory (DFT) calculations were performed. The proposed reaction coordinate diagram, and corresponding

free energies for the various intermediates and transition states for the reaction between complexes **1**, **2**, **3** and acetylacetonate - in SMD-methanol (continuum solvent model) are shown in Figure 5.

The computational studies explore the feasibility of an oxidative activation of the 2,3 carbon-carbon bond of acetylacetonate via a substrate-bound species, which displaces the labile water. Thermodynamically, this complex is favorable with respect to its water-bound parent by an average of 7.2 kcal/mol when considering all three complexes, consistent with NMR titration studies (Figure S11). The displacement energies for the NO₂-substituted complexes **2** and **3** are slightly more exergonic (blue and red values), consistent with the greater Lewis acidity they are proposed to impart to the nickel center. Computational analysis of a bidentate binding of substrate suggest that k¹- and k²-acac coordination modes (the latter being generated by displacement of a pyridyl arm) are essentially degenerate, implying that both linkage isomers may exist in equilibrium in solution. The rate limiting step of the modeled reaction passes through a transition state (**TS-1**, Figure 5) that is an association between the 2,3-carbon-carbon enolate bond of the bound substrate and dioxygen to form a peroxo type intermediate (**Int1**) with reasonable activation barriers of 19 – 23 kcal/mol, depending on the ligand substituents, consistent with a manageable activation barrier under mild temperature experimental conditions. The formation of the second intermediate, a 1,2 dioxetane complex (**Int2**), is preceded by intramolecular attack of the peroxo on the bound carbonyl carbon of the enolate. It is hypothesized that this step in the mechanism is facilitated by an intramolecular interaction between the distal oxygen in the peroxo intermediate and a hydrogen atom on the backbone of the ligand. This interaction starts with **TS1** and its observed in both **Int1** and **TS-2** (calculated bond lengths 2.11 Å, 1.76 Å, and 1.91 Å respectively). This interaction may be key to guiding the peroxo towards formation of the dioxetane (**Int2**). To further test this interaction, different conformational states of the substrate bound to the metal were explored and no other conformation yielded the observed H_L...O_{peroxo} interaction. The H_L...O_{peroxo} interaction is also intriguing as it could suggest secondary coordination sphere involvement and directing effects in the observed regioselective activation of the native substrate in the enzyme. The intermediates in the proposed mechanism such as the formation of a peroxo intermediate, and a 1,2 dioxetane have been documented for other model complexes.³¹ Finally, the dioxetane complex (**Int2**) collapses in a highly exergonic process to form pyruvaldehyde and an acetate bound species (**Prod**). The exothermicity of product formation observed in the calculations matches values from previous enzymatic,¹³ as well as biomimetic computational work¹⁸ in analogous substrates performing C-C bond cleavage. Evidence for the accessibility of an acetate bound product was published in earlier work,²³ and synthetically in this work via ¹H-NMR (Figure S13). Pyruvaldehyde, the other product of the reaction, is known to decompose further in the presence of oxygen to form another equivalent of acetic acid and carbon dioxide^{30b} consistent with experimental observations. As shown in Figure 5, similar computational results were observed for the two derivatives complexes **2** and **3** with slight differences in the calculated energies of intermediates and transition states, suggesting modest thermodynamic and kinetic impact on the reactivity imparted by the structural modifications.

The calculations were also scrutinized for evidence of redox activity at the metal center and the supporting ligand framework. Analysis of the spin density yielded no evidence of changes to the spin density of the nickel(II) center. For all supporting ligand types and all stationary points - ground and transition states - the calculated (Mulliken population analysis) - was uniformly $1.80(2) e^-$, consistent with a high-spin Ni(II) with a small degree of metal-ligand covalency. The only exceptions are, of course, the Ni-O₂ adducts, where spin density is delocalized onto the oxygen atoms of dioxygen, with more spin density on the terminal (non-coordinated) vs. the coordinated oxygen of the O₂ ligand, as expected from the classical Drago spin-pairing model of dioxygen binding.³² The calculations are thus consistent with cyclic voltammetry experiments that indicate highly cathodic redox potentials and no redox activity at the ligand.

While the outlined computationally derived oxygenation mechanism is of most relevance to biomimetic oxygenase activity, a hydrolytic/solvolysis retro-Claisen type reaction should also be considered since it has been reported in the literature that diketones such as acetylacetonate can undergo carbon-carbon hydrolytic retro-Claisen bond cleavage in the presence of metals³³ as well as other Lewis acids.³⁴ To our knowledge, no such reactivity has been reported with nickel complexes. The proposed hydrolysis could result from water attack on the bound carbonyl of acetylacetonate followed by formation of a diol, tautomerization, and final carbon-carbon bond cleavage resulting in the production of acetic acid and an acetone enolate that should rapidly protonate under the reaction conditions to yield acetone. Similarly, with methanol (reaction solvent) as the nucleophile this would result in the formation of methyl acetate and acetone. It is important to reiterate that no retro-Claisen products were observed experimentally under the aforementioned reaction conditions in either the aerobic or anaerobic regimes for any of the complexes. When the experiment was carried out under anaerobic conditions in D₂O no hydrolytic products were observed either, although the complex only displayed partial solubility in D₂O.

For purposes of further assessing the observed lack of a solvolysis/hydrolytic pathway under reaction conditions computational studies were performed to probe the feasibility of a hydrolytic mechanism (see Supporting Information). DFT results suggest that expected intermediates from the hydrolysis of the Ni-acac complex are > 20 kcal/mol uphill in free energy relative to the reactants in Figure 5, and so not competitive from a thermodynamic perspective.

As it was mentioned earlier the oxidative reactivity of unsubstituted diketone substrates by Nickel(II) complexes has been reported.^{21,22,35} The conclusions from this work showed that unsubstituted diketones e.g. acetylacetonate, are generally unreactive towards oxidative cleavage. Elsberg et al. showed the lack of oxidative reactivity of a collection of N3-tridentate Ni(II) unsubstituted diketone complexes.²² This they concluded is consistent with the literature which suggests the activation of diketones is only possible for electron rich diketone ligands. These results rebutted the findings of previous work by Ramasubramanian et.al.²¹ The observed reactivity and computational studies in this work suggest that an unsubstituted diketone ligand (acetylacetonate) is capable of undergoing oxidative cleavage when reacted with complexes **1–3**. These contrasting results point towards the key differences of the complexes in previous reports and our work. Complexes

1–3 have an N₄O ligand environment in contrast to all other examples with N₃ and N₄ chelates. It could be hypothesized that the presence of the oxygen donor in the chelating ligand might allow for activation of such substrates by imparting additional electron density to the nickel center supplanting the need for electron rich substrates. Also, it is important to point out that there are key differences in the reaction conditions between these studies and our work e.g. oxygenation times, temperature, presence or absence of water. We currently don't have enough experimental evidence to make a direct comparison for all the conditions. This current proof of principle study opens the door to suggest role that ligand environment might play in these substrate activations, but it also highlights the need for further studies that test the influence of ligand environments analogous to the enzyme and their influence on oxidative reactivity.

This research indicates the possibility that the model complexes in this study are capable of biomimetic carbon-carbon bond cleavage reactions. Additional kinetic and mechanistic labeling studies are ongoing. The preliminary observations presented here are promising and constitute the first example of N₄O-Ni(II) complexes displaying such reactivity, and are a needed addition to the ongoing biomimetic work relevant to ARD. Future work includes complete mechanistic studies of the reaction, further testing of oxygenation activity towards more closely structurally aligned substrates for ARD already published in the literature, ^{18,19,20} and probing the influence of ligand donating abilities on reactivity. These advances will contribute more directly to the debate over how the metal, substrate coordination, and electronic structure of the enzyme's active site impact the unique regioselectivity of ARD.

4. Conclusions

A novel family of structural and functional models of acireductone dioxygenase (ARD) were introduced and structurally characterized. This research, therefore, expands the availability of biomimetic models of an intriguing and important enzyme. In spite of structural differences between these models and the resting state of ARD, these complexes are the first to incorporate an oxygen binding moiety to the metal coordination environment, and a nickel-bound water, both analogous to that of the ARD resting state. Building on the structural characterization, the dioxygen mediated carbon-carbon cleavage of a model substrate (LiACAC) promoted by these Ni^{II}-N₄O complexes was explored experimentally and computationally. Experimental observations provide indirect evidence for the proposed mechanism that invokes substrate binding to nickel that provides a path for substrate activation via oxidation by dioxygen, in biomimetic fashion. The computationally supported mechanism invokes reactive intermediates observed in other dioxygenase model systems and enzymes with experimentally relevant barriers of activation. The observed reactivity, to our knowledge, is the first examples of biomimetic activity of an N₄O nickel system. Furthermore, the propensity of these complexes towards binding of substrate in two possible modes, mono and bidentate fashion, suggests a unique possible pathway for oxidative cleavage not yet reported in the literature of nickel oxidative biomimetic systems. Further work with human relevant metals (e.g., Fe and Co) will be necessary given the recent work in HsARD⁷ where monodentate substrate binding is proposed to undergo oxidative cleavage mediated by an oxygen-bound oxidant. Detailed mechanistic studies are underway in our laboratory to fully understand this observed oxidative reactivity. Additionally, we have

recently developed a related family of N₃O systems to allow for possible bidentate binding of substrate. With these complexes, whose synthesis, characterization, and reactivity will be reported in due course, we hope to expand in a systematic fashion understanding of oxygenases in general, and more specifically how acireductone dioxygenase might carry out its regioselective, metal-dependent, oxidative cleavage. Studying the mechanism of the complexes introduced here and derivatives thereof can thus contribute to answering many questions that remain with regards to ARD's mechanism of action and its involvement in human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A family of novel Schiff-base nickel complexes as models for oxygenases
- Proof of principle work studying biomimetic, nickel mediated, substrate activation
- Density functional theory derived proposed mechanisms

Figure 1.

Key features of the methionine salvage pathway. "On and off" ARD pathways display distinct product formation based solely on the identity of the metal center. Metal promiscuity studies show that both Mn and Co follow the "off pathway" route.

Figure 2.

Left: Active site of Ni-ARD (PDB ID: 5I91).⁴ Center: Skeletal structure of the active site.

Right: Skeletal drawing of the ARD model complexes presented in this work.

Figure 3.

Crystal structures of $[\text{Ni}^{\text{II}}(\text{O}^{\text{Ph}}\text{N}_4(6\text{-H-DPEN})(\text{H}_2\text{O}))](\text{OTf})$ (1), $[\text{Ni}^{\text{II}}(\text{O}^{\text{PhNO}_2}\text{N}_4(6\text{-Me-DPEN})(\text{H}_2\text{O}))](\text{OTf})$ (2), and $[\text{Ni}^{\text{II}}(\text{O}^{\text{PhNO}_2}\text{N}_4(6\text{-H-DPEN})(\text{H}_2\text{O}))](\text{OTf})$ (3). Thermal ellipsoids are drawn at the 50% probability level. H atoms and counterions are omitted for clarity.

Figure 4.

¹H-NMR in CD₃OD of the reaction mixture between **1** with LiACAC under O₂ (top); **1** and LiACAC under N₂ (Bottom).

Figure 5.

Calculated free energy profile for the oxidation of acac catalyzed by [Ni^{II}(O^{Ph}N₄(6-H-DPEN)(H₂O))]⁺ (**1**), [Ni^{II}(O^{Ph}NO₂N₄(6-Me-DPEN)(H₂O)](OTf) (**2**), and [Ni^{II}(O^{Ph}NO₂N₄(6-H-DPEN)(H₂O)](OTf) (**3**). Quoted values are free energies in kcal/mol and were derived using DFT as outlined in Computational Methods. Multiplicities for each species is indicated by the superscript to the left of the name. The product of the reaction (not pictured) is an acetate-bound Ni(II) species.

Scheme 1.

Synthetic procedure of biomimetic metal complexes **1** ($R=\text{H}$, $R_1=\text{H}$), **2** ($R=\text{CH}_3$, $R_1=\text{NO}_2$), **3** ($R=\text{H}$, $R_1=\text{NO}_2$) from ligand precursors LP1 and LP2.

Scheme 2.

Proposed reaction mechanism for the oxygenation LiACAC mediated by the Ni-N₄O complexes.

Table 1.

Key structural parameter comparisons for complexes **1**, **2**, **3** and ARD active site from Mm-ARD (PDB ID: 5I91).⁴

	1	2	3	Mm-ARD	
Ni-N1(N-tert.)	2.120(3)	2.098(2)	2.099(7)	Avg. Ni-N	2.1
Ni-N2(im)	2.001(3)	2.047(2)	2.010(7)	Avg. Ni-O(H ₂ O)	2.15
Ni-N3(Py-right)	2.104 (3)	2.159(2)	2.084(8)	Ni-O (Glu)	2.1
Ni-N4 (Py-left)	2.097(2)	2.184 (2)	2.087(8)		
Ni-O(Ph)	2.024(2)	2.001(2)	2.009(6)		
Ni-O(H ₂ O)	2.075(2)	2.125 (2)	2.139(6)		