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The metal drives the chemistry: Dual functions of acireductone dioxygenase

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Abstract

Acireductone dioxygenase (ARD) from the methionine salvage pathway (MSP) is a unique enzyme that exhibits dual chemistry determined solely by the identity of the divalent transition metal ion (Fe^{2+} or Ni^{2+}) in the active site. The Fe^{2+} containing isozyme catalyzes the on-pathway reaction using substrates 1,2 dihydroxy-3-keto-5-methylthiopent-1-ene (acireductone) and dioxygen to generate formate and the ketoacid precursor of methionine, 2-keto-4-methylthiobutyrate, whereas the Ni^{2+} containing isozyme catalyzes an off-pathway shunt with the same substrates, generating methylthiopropionate, carbon monoxide and formate. The dual chemistry of ARD was originally discovered in the bacterium *Klebsiella oxytoca*, but it has recently been shown that mammalian ARD enzymes (mouse and human) are also capable of catalyzing metal-dependent dual chemistry *in vitro*. This is particularly interesting since carbon monoxide, one of the products of off-pathway reaction, has been identified as an anti-apoptotic molecule in mammals. In addition, several biochemical and genetic studies have indicated an inhibitory role of human ARD in cancer. This comprehensive review describes the biochemical and structural characterization of the ARD family, the proposed experimental and theoretical approaches to establishing mechanisms for the dual chemistry, insights into the mechanism based on comparison with structurally and functionally similar enzymes and the applications of this research to the field of artificial metalloenzymes and synthetic biology.

Graphical Abstract

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Notes

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1. Introduction

Acireductone dioxygenases (ARDs) are a unique family of enzymes that exhibit different chemical and physical properties for the same polypeptide based solely upon the identity of the metal in the active site. If iron (Fe^{2+}) is bound, ARD catalyzes the penultimate step in the methionine salvage pathway (MSP), oxidatively generating the ketoacid precursor of methionine, 2-keto-4-(methylthio)butyric acid. However, if nickel (Ni^{2+}) is bound, an off-pathway product, carbon monoxide, is generated, along with 3-methylthiopropionic acid.¹ The off-pathway chemistry is supported by the Co^{2+} and Mn^{2+} reconstituted forms, as well. This dual chemistry was first identified for a bacterial (*Klebsiella oxytoca* originally identified as *K. pneumoniae*) ARD.² Recently it was shown that this dual ARD activity is also exhibited by mammalian enzymes (mouse and human ARD isozymes).^{3–4} In addition to its enzymatic function, several studies have indicated a role of ARD in carcinogenesis and tumor metastasis. ARD is also implicated in hepatitis C infection⁵, Down's syndrome (DS)-associated congenital heart defects (DS-CHD)⁶ and fecundity in *Drosophila*.⁷ In plants, ARD expression is associated with development and fruit ripening, and so is of great interest to plant biologists.⁸ ARD is likely a multi-functional (“moonlighting”) enzyme involved in both regulatory and enzymatic functions. The field of ARD research has seen significant progress over the past 10 years. The goal of this review is to describe this remarkable enzyme family, including the mechanistic and functional aspects of the dual chemistry and the implications of this research on the fields of metalloproteins, disease progression and synthetic biology.

ARDs belong to a structural class known as cupins (from the Latin *cupula* for “small barrel”), which are formed from several turns of antiparallel β -sheet into a β -helix, a motif also called a “jellyroll”. The cupin motif typically exhibits asymmetry, with one end of the β -helix more “closed” and the other more “open”. The active sites of cupin enzymes are typically found in the open end. Many other non-heme iron-dependent oxygenases, including oxalate decarboxylase and oxalate oxidase, share the cupin motif, as do most ketoglutarate-dependent oxidases (Figure 1).^{9–12}

Biological implications of ARD functions

The observation of off-pathway chemistry with mammalian ARD enzymes is particularly interesting in that carbon monoxide (CO) has both signaling and anti-apoptotic properties *in vivo*.¹³ We suspect that ARD gain-of-function as a result of off-pathway chemistry may be a

novel source of CO in the cell (currently heme oxygenase is the only confirmed source of biogenetic CO in normal human tissue).¹⁴ Also interesting is the observation of ARD gene up- (and down-) regulation in a variety of carcinomas,¹⁵ along with the evidence that ARD may regulate matrix metalloproteinase MT1-MMP, which is associated with tumor metastasis.¹⁶ A search of the NCBI GEO gene expression profiles for *ADII*, the gene that encodes ARD, yields several hundred comparisons showing either up- or down-regulation of this gene in cancer cell lines compared with normal tissue or when treated with a chemotherapy agent. This leads to the intriguing possibility that the off-pathway chemistry catalyzed by the Ni²⁺, Co²⁺ or Mn²⁺-bound enzyme may be relevant in pathology in mammals. The binding of non-ferrous divalent metals in human ARD active site could help protect tumor cells from apoptosis by producing CO. Ni is toxic in mammals, and to date no native Ni-binding metalloenzyme has been identified in mammals.¹⁷

Mechanistic considerations

There have been inorganic models¹⁸ and computational studies¹⁹ of the mechanism of the metal-based functional switching in ARD, but to date there is no conclusive evidence precluding any of the proposed mechanisms. In order to understand how the metal can modulate the function of the enzyme, we have characterized structures of both the bacterial and mammalian ARD enzymes.^{3, 20–21} Published structures of enzyme-substrate analog and enzyme-product/product analog complexes provided insights into the mechanistic details of the enzyme.³ A comparison of ARD to structurally and functionally similar cupin enzymes provided further insights into potential mechanisms that may help rationalize dioxygen activation that could not be explained by the postulated mechanisms.

Metal sensing in biological systems

To date, only the bacterial ARD isozymes from *K. oxytoca* have been characterized structurally with Fe and Ni metals bound to the enzyme.^{20–21} While both structures show the same basic cupin fold, repacking of hydrophobic cores upon metal replacement result in displacement and disruption of helices and a profound change in active site accessibility. Preliminary results on the human ARD enzyme suggest that these same changes are observed in eukaryotic ARDs as well.⁴ These changes take place despite the fact that the same protein-based ligands are used to bind both Fe²⁺ and Ni²⁺ in the bacterial enzyme, and both ions adopt the same pseudo-octahedral geometry in the active site. As such, the ARD isozymes provide a remarkable example of how two metal ions of similar size and identical charge can be distinguished by a folded protein. Understanding how these structural changes are propagated through the protein structure has obvious implications for a vast array of biological machinery that must make such distinctions: these include metallochaperones, metal-dependent response factors and transcription regulators and ion channels, as well as the more obvious examples of metalloenzymes.

Enzyme engineering

In enzymes requiring a metal cofactor for activity, the replacement of the native metal with an abiological metal provides unique opportunities for enzyme engineering. A recent study has reported replacement of iron in Fe-porphyrin proteins with abiological noble metals to create enzymes that catalyze reactions not duplicated in nature.²² Understanding the ARD

dual chemistry sets the stage for exploring the role of metals in biocatalysis to create artificial metalloenzymes that perform novel reactions. As we and others have observed, metals can not only modulate the reactivity of a metalloenzyme, but can also expand the scope of reactions catalyzed by native metalloenzymes.

2. The methionine salvage pathway

The methionine salvage pathway (MSP) is ubiquitous in plants, animals and bacteria.²³ The MSP recycles the terminal thiomethyl group of methionine, an essential amino acid, and regulates the concentration of several metabolic intermediates in the pathway. In the first step of the MSP, S-adenosylmethionine (SAM) is formed enzymatically from methionine and ATP (Figure 2). SAM is a critical source of electrophilic methyl and ethylene groups in biosynthetic processes, and is involved in the synthesis of polyamines, spermine and spermidine in animals and ethylene in plants. Polyamines are required for cell growth and proliferation²⁴ while ethylene is required for ripening of fruits and vegetables.²⁵ Defects in polyamine regulation are associated with defects in cell cycle, cell growth and DNA replication. Inhibition of polyamine synthesis arrests DNA replication,²⁶ and their synthesis is downregulated in senescent and mature tissue.²⁷ Elevated polyamine levels are associated with suppression of apoptosis and tumor formation.^{28–29} Neoplastic cells from colorectal cancers are found to have higher levels of polyamines than normal tissue.³⁰ Methylthioadenosine (MTA) is formed as a byproduct from SAM during polyamine synthesis in animals and ethylene synthesis in plants, and is the first committed intermediate in the MSP.^{24, 31} MTA is an inhibitor of both polyamine synthesis and transmethylation reactions,^{24, 32} so intracellular levels of MTA are tightly regulated. The MSP is critical in maintaining MTA homeostasis by returning it through a series of reactions to methionine, thereby “salvaging” the thiomethyl group of the original methionine.³³ As such, this pathway is sometimes referred to as the MTA cycle. ARD catalyzes the penultimate step in the MSP, the oxidative decomposition of substrate acireductone (1, 2-dihydroxy-3-keto-5-(thiomethyl) pent-1-ene) to formate and 2-keto-4-(thiomethyl) butyrate (KMTB), the keto-acid precursor of methionine. The ARD substrate acireductone is formed by the action of E1 enolase phosphatase, a member of the haloacid dehalogenase superfamily, on 1-phosphonoxy-2, 2-dihydroxy-3-oxo-5-methylthiopentane (E1 substrate).

3. ARD from *Klebsiella oxytoca*

The first ARD to be identified and structurally characterized, KoARD, was isolated from the bacterium *Klebsiella oxytoca*. The enzyme (referred to in the early publications as “E2”) was identified by the Abeles group in the course of mapping the MSP in that organism. It was noted that ¹⁴C labeling of methionine resulted in the formation of radiolabeled methylthiopropionate (MTP), suggesting that a previously unknown shunt reaction from the MSP was occurring.^{34–36} That group later identified both the on- and off-pathway activities as the result of a single polypeptide with different physical and catalytic properties depending upon the metal bound to the enzyme, referred to by those workers as “one protein, two enzymes”.¹ They found that this enzyme exhibits different activity depending on the identity of the metal ion cofactor. KoARD with Fe²⁺-bound (“E2’”) was found to catalyze on-pathway MSP chemistry leading to production of formate and the keto-acid

precursor to methionine, whereas Ni²⁺-bound KoARD (E2) catalyzed an off-pathway shunt leading to production of carbon monoxide, formate, and 3-(methylthio) propionic acid (MTP).^{1–2} Both the Fe²⁺ and Ni²⁺-bound forms of the protein were isolated from *Klebsiella*,³⁵ and later also obtained upon overexpression in *E. coli*. Both forms of the enzymes are monomers and co-elute during size exclusion chromatography, but can be separated by ion exchange or hydrophobic interaction chromatography, suggesting that the differences between the two isoenzymes were structural rather than due to differences in oligomerization state. KoARD shows tighter binding to Ni²⁺ compared to Fe²⁺ since Fe can be removed from the folded enzyme by dialysis with EDTA, while Ni requires denaturation before EDTA dialysis for removal.³⁷ Addition of equimolar amounts of Fe²⁺ and Ni²⁺ to the apoenzyme produced > 80% of Ni-containing form within a minute. The activities of the two enzymes could be interconverted by exchanging Fe²⁺ and Ni²⁺.³⁷ The enzyme was also found to be promiscuous, with Co²⁺ and Mn²⁺ give Ni-like activity and Mg²⁺ yielding a low level of Fe-like activity (although the possibility of trace Fe²⁺ contamination of Mg²⁺ cannot be discounted).³⁷ The Ni²⁺ and Fe²⁺-bound KoARDs represented the only known pair of naturally occurring metalloenzymes with distinct chemical and physical properties determined solely by the identity of the metal ion in the active site. The purpose of the off-pathway reaction catalyzed by Ni²⁺-bound ARD in *K. oxytoca* is still unknown. The prevalence of Ni²⁺-bound ARD in native *K. oxytoca* suggests that it may be physiologically necessary and may be involved in the regulation of the MSP, or that CO production by *Klebsiella* has a symbiotic role in wild ecosystems.^{38–40} CO is a substrate for carbon monoxide dehydrogenase, which catalyzes the reversible oxidation of CO to CO₂, which could allow symbiotes (or *Klebsiella*) to use carbon monoxide as a source of reducing equivalents.^{41–43} CO is also known to be a signaling molecule for a transcriptional regulator, CoxA, in bacteria.⁴⁴ CO production from methylthioribose has also been reported for *Bacillus subtilis*, indicating the presence of a stable off-MSP shunt.⁴⁵

A detailed analysis of enzyme kinetics found no evidence for a stable M²⁺-O₂ complex in either the Fe or Ni isoforms of KoARD.³⁷ The same paper described the use of model substrates to probe mechanism, with a convenient synthetic route described separately (Scheme 1).⁴⁶ Spectroscopic evidence suggested that the initial enzyme-substrate complex was a chelate between the metal and the dianion **5e**. Based upon slow inactivation of the enzyme by suicide substrate **5b** it was proposed that the reaction proceeds by a one-electron transfer from substrate to O₂, generating the peroxide radical species **6** (Scheme 2).

The feasibility of a peroxo intermediate in Ni-bound KoARD was tested by reaction of hydrogen peroxide with several model compounds. The symmetric β-diketone **6** was shown to form the cyclic peroxide **7**, and the bis-hydroxy compound **8** decomposed upon treatment with hydrogen peroxide to form two equivalents of acetic acid along with a molecule of CO (Scheme 3).⁴⁴

4. Structures of KoARD isoforms and structural differences arising from differential metal binding

The first ARD structures to be determined were those of the Ni-KoARD (PDB ID: 1ZRR) and Fe-KoARD (PDB ID: 2HJI), both by solution NMR spectroscopy.^{20–21, 47} ARD belongs to the cupin superfamily, which is characterized by a conserved β -barrel (the “cupin barrel”) consisting of anti-parallel β -sheets that gives rise to a classic β -helix “jelly roll” motif. Due to the paramagnetism of the metal ion, the active site of the Ni²⁺-bound KoARD could not be characterized directly by NMR, and so was modeled using the structure of another cupin, jack bean canavalin, with the model supported experimentally by X-ray absorption spectroscopy (XAS).²¹ The structure of the Fe²⁺-bound KoARD is a model based on the structure of a stable soluble metal-free mutant of ARD, H98S, which is isostructural with Fe-KoARD as determined by a comparison of ¹D ¹H NMR, NOESY and 2D ¹H, ¹⁵N heteronuclear single quantum coherence (HSQC) spectra.²⁰

Remarkably, it was observed that the same four protein residues were involved in ligation, regardless of the identity of the metal ion bound. These protein-derived ligands include three histidine (His) residues, His96, His98 and His140 and one glutamate (Glu102), all of which were later found to be strictly conserved in other ARD homologues. This was surprising, in that it might be expected that the very different chromatographic and enzymatic behaviors of the Fe- and Ni-bound forms of ARD, both monomers, could result from different ligands or a change in the number of ligands used for different metals. However, mutation of any of the conserved ligand residues in KoARD resulted in inactive enzymes that did not bind any metal. Furthermore, the XAS data for both the Fe- and Ni-bound KoARD isozymes supported similar distorted octahedral geometries for both metal ligation spheres.⁴⁸

Given that the Ni²⁺- and Fe²⁺-bound forms of bacterial ARD make use of the same ligand set, the reasons for the chromatographic separability and different thermal stabilities of the isozymes must be sought elsewhere. As can be seen from Figure 3, the most readily observed structural difference between the two forms is the C-terminal 3₁₀ helix (**Q** in Figure 3) forming a cap on top of the β -barrel in the Ni-bound enzyme, whereas the same region of the polypeptide is disordered in the Fe-bound form, which is missing secondary structural features **O-Q**. **O** is a single turn of helix, and **P** is a short additional strand edge to the β -barrel. The loss of these features renders the Fe-bound KoARD active site significantly more accessible and open to solvent than in the Ni form. Also, the **E,F** helices on the opposite face of the barrel pivot by ~20° between the Fe- and Ni-bound ARD isoforms. This results in a significant repacking of a secondary hydrophobic core on that face of the barrel, as well as a change in the order of a loop of polypeptide **D** at the N-terminal end of the **E** helix system. In the Ni²⁺-bound enzyme, this loop is largely disordered, while in the Fe²⁺-bound form, it becomes more structured. We refer structural entropy switch: In the Ni-bound ARD, the C-terminus is ordered, while the **D** loop is disordered. In the Fe form, the opposite situation is observed.²⁰ The origins of these conformational shifts are not obvious, but we have hypothesized how they might be driven. While structural entropy switch: In the Ni-bound ARD, the C-terminus is ordered, while the **D** loop is disordered. In the Fe form, the opposite situation is observed.²⁰ The origins of these conformational shifts are not obvious,

but we have hypothesized how they might be driven. While to this phenomenon (increased order in one location with decreasing order at another) as a 0251681792 both Fe^{2+} and Ni^{2+} are nearly identical in size ($r \sim 70$ pm), the XAS data indicate that the Ni-N/O bond lengths in ARD are on average ~ 0.1 Å (10 pm) shorter than the corresponding bonds to Fe. While this is not a large change, the fact that three of the four protein-based ligands (His96 on the edge strand following helix **H**, Glu102 on strand **I** and His140 on strand **M**) originate within the β -barrel, and are thus restrained from changing position as a result of the shorter bond lengths in NiARD, the bond shortening may be amplified by pulling the Ni deeper into the barrel mouth, dragging the fourth ligand (His98) closer to the barrel opening than it is in the Fe^{2+} -bound form, and stabilizing the loop containing His98 as an edge of the β -barrel. This in turn results in the formation of the short antiparallel β -strand **P** between the C-terminal loop and the new barrel edge. As a result, the active site of the Ni^{2+} -bound form is considerably less accessible than that of the Fe-bound enzyme, as shown in Figure 4. Note that Trp162, which is disordered in the Fe enzyme, is ordered and close to the metal center in the Ni form. Phe92 is also more distant from the metal center in the Fe-KoARD than in the Ni isozyme. This rationalizes the observation that Fe^{2+} can be removed from the active site relatively easily with chelating agents, while the Ni^{2+} form must first be denatured to remove the metal, and furthermore explains the significant increase in thermostability observed upon Ni^{2+} binding in all ARD enzymes characterized to date.^{3–4} This hypothesis, while not fully tested, also provides some insight into how metal selectivity might be achieved in other metal binding proteins, including transporters and regulatory transcription activators and repressors, with only standard amino acid-based ligands.

5. ARD homologs from plants and *Drosophila*

ARD has now been characterized from several eukaryotes. The MSP from plants has been studied extensively, as MTA is a byproduct of ethylene biosynthesis. Ethylene is a hormone involved in fruit and vegetable ripening. The *OsARD1* gene from rice (*Oryza sativa*) was identified as a primary ethylene response gene.⁸ The expression of this gene is strongly up-regulated at low ethylene levels as an early response to ethylene production under hypoxic conditions resulting from submergence. Recombinant Fe^{2+} bound-*OsARD1* protein is a trimer, and performs on-pathway MSP chemistry. The Ni^{2+} -reconstituted *OsARD1* exhibits off-pathway chemistry albeit with much lower activity than Fe-*OsARD1*, and polymerizes beyond the trimeric state. For these reasons, Ni-*OsARD1* is deemed unlikely to represent a physiologically relevant form of ARD in rice. A second gene *OsARD2* was also identified which was constitutively expressed and unlike *OsARD1*, its expression was not induced by deep water submergence. *OsARD2* has an 85% identity and 93% sequence similarity to *OsARD1*.

ARD from *Arabidopsis thaliana* (AtARD1) was identified as an effector of G β (AGB1) which is a subunit of the heterotrimeric G protein complex. The activation of G protein complex promotes interactions with other proteins during cell signaling and communication to the cytoplasm. *Arabidopsis* plants lacking the AGB1 mRNA transcript have several abnormal developmental phenotypes, including aberrant leaf shape, increased root mass, silique morphology, and hypersensitivity to infection.^{49–51} AtARD1 expression suppresses G β -null mutant phenotypes and controls embryonic hypocotyl length by modulating cell

division.⁵² As with other ARD homologues, recombinant Fe²⁺-bound AtARD1 catalyzes on-pathway MSP chemistry. Enzymatic studies of AtARD1 in the presence and absence of AGB1 showed that ARD enzymatic activity is stimulated by AGB1 *in vitro*. In addition to *AtARD1*, the *A. thaliana* genome encodes *AtARD2*, *AtARD3* and *AtARD4*. The roles of these other gene products are unknown at this time, nor is there any information regarding the conditions under which they are induced (or even if they are induced at all).⁵² However, comparison of the results for rice and *A. thaliana* suggests that many plants have more than one ARD gene.

A role of *Salvia miltiorrhiza* (red sage) acireductone dioxygenase (SmARD) gene in the defense response under different abiotic stressors like drought, cold and salt has been found.⁵³ Similarly, wheat (*Triticum aestivum*) ARD (TaARD)⁵⁴ and potato (*Solanum tuberosum*) ARD (StARD)⁵⁵ have been shown to be involved in the defense response against stressors such as wounding. ARD is likely involved in ethylene synthesis and ethylene signaling in response to biotic and abiotic stresses in these plants as well.

The presence of a functional ARD gene product has been shown to be required for normal fecundity in *Drosophila*.⁷ In dietary restriction conditions, the egg production of acireductone dioxygenase 1 (*ADII*) mutant flies was reduced compared to that of control flies. This fecundity defect in mutant flies was rescued by either methionine supply or reintroduction of *ADII* gene. A functional homolog of human *ADII* was also able to rescue the fecundity defect.

6. Mammalian ARD homologs and “moonlighting” functions of ARD: Links to carcinogenesis, congenital defects and viral susceptibility

Mammalian ARDs from mouse (MmARD) and human (HsARD) have now been investigated both biochemically and structurally in order to relate metal ion identity and three-dimensional structure to enzyme function.³⁻⁴ Both MmARD and HsARD have been shown to perform the same metal-dependent dual chemistry *in vitro* as observed with KoARD.^{1, 3-4} Unlike the bacterial forms, where both isoforms displayed similar turnover rates, the Fe²⁺-bound forms of the mammalian enzymes showed several-fold higher activity than the Ni²⁺, Co²⁺ or Mn²⁺ forms. However, like KoARD, the Fe²⁺-bound forms catalyze on-pathway chemistry, while the Ni²⁺, Co²⁺ and Mn²⁺ forms catalyze off-pathway chemistry. Also, as with KoARD, the thermal stability of these mammalian enzymes is a function of the metal ion identity, with Ni²⁺-bound enzyme being the most stable, followed by Co²⁺ and Fe²⁺, and Mn²⁺-bound enzyme being the least stable.³⁻⁴

The human ARD homologue HsARD has been shown to serve regulatory functions in addition to its enzymatic function in the MSP. Seiki *et al.* showed that HsARD binds to the cytoplasmic tail of membrane-type 1 matrix metalloproteinase (MT1-MMP) and inhibits MT1-MMP-mediated cellular invasiveness.¹⁶ A recent study by Pratt *et al.* has indicated a role of HsARD in regulating the intracellular function of MT1-MMP-mediated autophagy in brain tumors.⁵⁶ In this study, an inverse correlation was observed between the expression levels of ARD and MT1-MMP in clinically validated grade I to grade IV brain tumor tissues. The functional impact of ARD on MT1-MMP mediated autophagy signaling was

further tested in glioblastoma cells. Using FRET microscopy and surface plasmon resonance it was shown that the cytoplasmic domain of MT1-MMP and ARD interacted with a dissociation coefficient of 12 μ M.⁵⁶ Oram *et al.* have shown that *ADII* encoding ARD (GenBank ID: 55256, *ADII*, 5 exons on chromosome 2, NC_000002.12, 3497919.3519579, complement) is down-regulated in rat prostate and human prostate cancer cell lines and its enforced expression induces apoptosis.¹⁵ These researchers have also shown that cultured gastric carcinoma cells and fibrosarcoma cells have downregulated *ADII*.^{15, 57} A recent clinical study testing the impact of environmental carcinogens on gene expression differences in humans found *ADII* as a gene having a direct link to cancer development.⁵⁸ An N-terminal truncated variant of HsARD called SipL was shown to be implicated in the replication of hepatitis C virus in non-permissive cell lines. At the time of publication of that work, the enzymatic function of the protein was unclear, and so it was identified as SipL (submergence-induced protein like) due to sequence homology with what was later identified as OsARD. This truncated version of HsARD has the first 63 amino acids missing.⁵ Based on structural homology with KoARD, this deletes the N-terminal B helix and the edge strands A, C and D from the narrow end of the cupin barrel (Figure 3), but leaves the metal binding site and the major part of the barrel intact. On the other hand, MmARD and HsARD share an 86% sequence identity, and assuming that the SipL variant folds similar to the full-length MmARD, the entire the β -barrel is present in the SipL variant. It is unknown if SipL is a metal-binding protein or if it exhibits enzymatic activity.

A recent clinical study related the two known functions of HsARD. This study showed that the physical interaction between MT1-MMP and *ADII* led to suppression of HCV infection and this inhibitory effect could be reversed by *ADII* overexpression.⁵⁹ A recent study to identify the pathways disrupted in Down's syndrome (DS) and DS-associated congenital heart defects (DS-CHD) has indicated that *ADII* has higher expression in fetuses trisomic for Hsa21 than in normal fetuses. Based on this finding, the authors predicted that the methionine salvage pathway is significantly altered and could function as an indicator of DS-CHD.⁵⁶

6.1. Mammalian ARD structures

The first crystallographic structure determined for an ARD was that of the mouse homolog MmARD (PDB ID: 1VR3), published by the Joint Center for Structural Genomics (JCSG). However, the identity of the metal ion cofactor in that structure was not determined.⁶⁰ Recently, low resolution structures of ARD from *Bacillus anthracis* (2.97 Å) (PDB ID: 4QGM) and HsARD (3.05 Å) (PDB ID: 4QGN) have been deposited (Figure 5). The complete details of these structures have not been published and it is unknown if the proteins used in these structures were enzymatically active, nor have the identities of the metals in the active sites been confirmed.^{61–62} In the *B. anthracis* ARD (BaARD) structure, the bound metal is proposed to be Cd^{2+} , while in the HsARD structure, the bound metal ion is proposed to be Fe^{3+} . This is somewhat surprising, in that in our hands, oxidation results in the loss of metal and inactivation of the enzyme. Nevertheless, in all of these structures, the strict conservation of the coordinating ligands originating from the protein was confirmed (Figure 8).

We have recently described the crystallographic structures of Ni²⁺-bound (PDB ID: 5I91) and Co²⁺-bound MmARD (PDB ID: 5I8Y) (Figure 6) and found the structures of the two proteins to be similar (RMSD=0.06Å).³ Both Ni and Co exhibit octahedral co-ordination geometry in the ARD active site, using the N_ε atoms of imidazole protein ligands His88, His90, His133 and monodentate coordination by the carboxylate of Glu94 (Figure 6). In addition to the protein ligands, there are two distinct water (or hydroxide) ligands bound to the metal ion center, whereas the previously reported structure of MmARD showed undefined electron density in the corresponding regions. This is consistent with the NMR solution structure of Ni²⁺-bound KoARD in which two water molecules were modeled as ligands to fit EXAFS data in addition to the protein-based ligands.²¹

Ni-MmARD was crystallized with on-pathway product 2-keto-4-(methylthio) butyric acid (KMTB) (PDB ID: 5I91) (Figure 7) and off-pathway product analog valeric acid (VA) (PDB ID: 5I8S). Both KMTB and VA are found in the active site. Neither compound ligates the metal, but both are within hydrogen bonding distance of the two water/hydroxide ligands bound to the metal as well as the guanidinium group of Arg96, which is strictly conserved in all known ARD sequences. Both ligands have non-polar contacts with Phe135, Phe105, Phe84 and Ala145 (all of which are also strictly conserved as well as with Val143 and Ile98 (Figure 8). Obviously, a substrate-bound ARD structure would be desirable for mechanistic insights. However, acireductone is highly sensitive to oxidation, and to date this has not been accomplished. We attempted to incorporate substrate into the ARD structure, by generating desthio-acireductone *in situ* in an anaerobic cuvette, and adding this to a drop containing a Ni-MmARD crystal under anaerobic conditions. While the crystals diffracted and a structure was determined, no ligand was detected in the active site.³

We were able to obtain a structure of substrate analog D-lactic acid (D-LA) bound to Ni-MmARD solved to a resolution of 1.7 Å (PDB ID: 5I8T). D-LA was present in the active site and, unlike the product KMTB and product analog VA, D-LA was seen to coordinate directly to the metal (Figure 7). The two water molecules bound to the metal ion in the KMTB-bound or VA-bound protein structures were replaced by one D-LA carboxylate oxygen and the D-LA hydroxyl oxygen, forming a five-membered ring equatorial chelate complex with the Ni²⁺. The carboxylate oxygen of D-LA not ligating the metal is within hydrogen bonding distance of N^ω atom of Arg96.³ D-LA has a similar arrangement of oxygen atoms as acireductone, but lacks the hydrophobic (methylthio) ethylene moiety of the native substrate. The recently deposited structure of Fe³⁺-HsARD (4QGN) has L-selenomethionine in the active site and it is coordinated to the metal ion in a manner similar to that of D-LA.⁶²

6.2 Comparison of NMR and crystallographic approaches to ARD structures

The fact that NMR was used to determine the original KoARD structures reflected the difficulties encountered in crystallizing KoARD rather than a preference for any particular methodology. Still, it is fortunate that the Fe²⁺- and Ni²⁺-bound KoARD isoforms are readily distinguishable spectroscopically: Their peripheral structures are quite different, particularly in the C-terminal regions, D loops and E, F helices of the two structures (see Figure 4). These differences have allowed us to make some structure-based hypotheses as to

the origins of both the physical and functional differences between the two isoforms. On the other hand, the NMR structures suffer from a lack of clarity regarding the precise structures around the (paramagnetic) metals in both forms of KoARD. While we have described NMR methods for making sequential resonance assignments in these regions,^{20–21, 47} standard NMR structural restraints (nuclear Overhauser effects, residual dipolar couplings and chemical shifts) are still lacking. For detailed structural investigations of metal binding geometry and arrangements of side chains in the active sites of ARD enzymes, crystallographic structures are essential. Because both of the recently published structures of MmARD with defined metal occupancies are of enzymes that exhibit off-pathway activity (Co^{2+} and Ni^{2+}), there is still little clarity regarding any structural differences between the Fe^{2+} and Ni^{2+} forms of the mammalian enzymes and how these relate to on- versus off-pathway activity in the MSP. Recently we have shown using solution NMR spectra of ^{15}N -labelled Fe^{2+} , Ni^{2+} and Co^{2+} -bound HsARD isozymes that, like KoARD, the different metals impart significant structural differences to the overall protein structure.⁴ Preliminary analysis of multidimensional NMR spectra of Fe-HsARD indicates that, like Fe-KoARD, the C-terminal polypeptide is largely disordered.⁴

7. Proposed enzymatic mechanisms for ARD

Enzyme kinetic studies of both Fe^{2+} and Ni^{2+} -bound forms of KoARD indicate a sequential mechanism where both acireductone and oxygen must bind to the enzyme prior to product release.³⁷ Spectroscopic analysis indicates that oxygen does not bind ARD in the absence of substrate. Acireductone first binds to the enzyme as a di-anion followed by oxygen binding to form a ternary complex.³⁷ Since the acireductone slowly reacts with oxygen non-enzymatically to give Fe-KoARD like products, it is possible that the redox nature of metal and metal-oxygen activation may not be important. Neither Ni-KoARD nor Fe-KoARD exhibit an EPR signal under aerobic or anaerobic conditions, nor in the presence or absence of the substrate acireductone. Both Ni^{2+} and Fe^{2+} are expected to have integer spins ($S=1$ and $S=2$, respectively), and hence EPR signals are difficult to observe due to the forbidden nature of the transitions. Still, the lack of any O_2 effect on either EPR or NMR spectra suggest that if redox activity is required at all, it may be transitory.³⁷ Since acireductone binds the metal as a dianion, the metal likely activates the substrate by acting as a Lewis acid. The following sections discuss various approaches to understanding the mechanism(s) of ARD activity.

7.1 Chelate Hypothesis

The chelate hypothesis postulates that different ARD chemistries arise from differences in the nature of the cyclic peroxide formed at the metal center. A five-membered ring involving coordination of C-1 and C-2 oxygen atoms of acireductone would form in the case of Fe-KoARD, while a six-membered ring involving C-1 and C-3 oxygen atoms is formed in the case of Ni-KoARD (Scheme 4). The NMR structures reveal a difference in the active site tertiary structure with Ni-KoARD featuring a closed form and Fe-KoARD showing an open form of the structure.²⁰ Docking studies show that the substrate acireductone could coordinate via a five-membered chelate in Fe-KoARD, but in the more congested Ni-ARD active site, it would bind via a less sterically demanding six-membered chelate ring.²⁰ These

binding modes would activate acireductone at the C1 and C2 positions in Fe-KoARD but at the C1 and C3 positions in Ni-KoARD, leading to on-pathway and off-pathway reactions, respectively. The chelate hypothesis is compatible with ^{18}O and ^{14}C incorporation in the products as determined using $^{18}\text{O}_2$ and ^{14}C -labelled acireductone.^{20–21, 37}

7.2 Small molecule models of ARD active sites

Berreau *et al.* have synthesized small molecule models of the metal centers of ARD in order to investigate possible mechanisms for ARD dual chemistry. They challenged the chelate hypothesis, showing that both Ni and Fe can form six-membered ring complexes with substrate homologues and give Ni-like products in anhydrous solvents. The on-pathway chemistry is observed with the Fe-center only in the presence of water. They attributed this to the hydration of the tri-ketone reaction intermediate formed in the first step of the reaction.^{19–20, 63} The working hypothesis is that the hydration of the triketone intermediate is mediated by Fe, but not by Ni. The crystal structures of the solvate complexes are pentacoordinate when $M = \text{Fe}$, with a single solvent molecule bound, but hexacoordinate with two solvent molecules when $M = \text{Ni}$ (Schemes 5 and 6). This differing coordination preferences of Fe and Ni may be responsible for the differences in Lewis acid activation of the triketone intermediate. These results are compatible with the fact that the Ni-KoARD active site is less accessible to solvent than the active site in Fe-KoARD.⁶⁴

7.3 Computational studies

Sparta *et al.* examined the mechanisms of ARD used mixed quantum-classical molecular dynamics simulations coupled with density functional theory. Their calculations show that, if the conserved Arg is included in the QM region of the simulations, a six-membered chelate of the metal by the substrate has the lowest energy in the cases of both Ni^{2+} and Fe^{2+} . As a result of these calculations, the differences in the chemistry are attributed to the redox-active nature of Fe^{2+} relative to Ni^{2+} , allowing Fe-ARD to form an intermediate partially stabilized by charge transfer to Fe^{2+} , whereas Ni simply acts as a Lewis acid (Scheme 7).¹⁹ While these calculations do not account for the non-enzymatic and Mg^{2+} -ARD catalyzed oxidative decomposition of acireductone, which give Fe-ARD like-products, there has not been a systematic investigation of either of those reactions: In particular, it is not known whether trace metals (in particular, iron) are responsible for the observed reactivity in either case. As such, the results of the computational work cannot be disregarded.

7.4 Mechanistic implications from crystallographic results

The crystallographic structures of Ni-MmARD and Co-MmARD provide the first detailed structural information regarding the mode of product binding and the possible modes of substrate binding in the active site of Ni^{2+} or Co^{2+} -bound MmARD. As noted above, D-lactic acid (D-LA) was found to chelate the metal centers so as to form a five-membered ring, as proposed in the chelate hypothesis, demonstrating that such structures are stable. Of course, the five-membered ring is an intermediate in the on-pathway reaction according to the chelation mechanism, rather than the off-pathway reaction catalyzed by the Ni or Co-bound forms in which this was observed. Still, the fact that the five-membered chelate is

observed suggests that, for now, none of the proposed mechanisms can be eliminated from consideration.

Other mechanistic insights can be gleaned from the crystallographic structures. For example, Arg96 is a strongly conserved (Figure 8) active site residue and is within hydrogen bonding distance of both substrate analog and product. (All residue numbers refer to the MmARD sequence). It has been suggested that this conserved Arg serves as a general base for deprotonation of substrate upon binding.³ While the guanidine functionality is usually too basic to serve as a general base in enzymatic reactions ($pK_a \sim 12.5$), the side chain of Arg147, also strongly conserved, is parallel to the side-chain of Arg 96, and so may modulate the pK_a of Arg96 sufficiently so that the residue can act as a general base at physiological pH.¹⁷ This parallel arrangement is also seen in the KoARD active site (Arg104 and Arg154, in the KoARD sequence). Other conserved residues in the active sites of ARD orthologs include Phe135, Phe105, Phe84 and Ala145. It is likely that these residues are involved in correct substrate orientation. Previous studies on KoARD have shown that the conserved tryptophan residue Trp162 (KoARD sequence) is ordered and adjacent to the active site entrance in Ni-KoARD, but disordered in the Fe-bound form. It was proposed that the side chain indole of Trp162 drives a preference for the binding mode leading to off-pathway products in Ni-KoARD, and its absence in the Fe form allows for a more sterically demanding mode leading to the on-pathway products.²⁰ A mutant of the analogous Trp residue in *Arabidopsis* homolog of ARD (AtARD1) (Trp166) was tested for enzymatic activity in the Fe-bound form and it was found that this mutant (W166A) was a more efficient enzyme than the wild-type AtARD1 enzyme, suggesting that the Trp may serve a gating function, reducing on-pathway activity when it occludes the active site.⁵² In the MmARD structure, this residue (Trp155) also resides in the loop region preceding the long C-terminal helix, and may be involved in conformational changes upon substrate binding (Figure 9). Phe149, also a conserved residue in this loop region may be involved in assisting Trp155 in conformational gating.

8. Enzymes structurally and functionally similar to ARD

The ARD enzymes are similar to other cupin superfamily members, having a characteristic β -barrel structure where the active site of the protein is located. The His₃-Glu₁ metal binding motif found in ARD is also seen in other cupin enzymes, including in oxalate oxidase, oxalate decarboxylase and quercetinase. Scheme 8 shows the reactions catalyzed by these enzymes.

As can be seen, of the three enzymes in Scheme 8, only QueD is a dioxygenase, while OxDC catalyzes the disproportionation of oxalic acid under acidic conditions to yield formic acid and CO₂, OxOx catalyzes the two-electron reduction of O₂ to hydrogen peroxide with concomitant oxidation of oxalate to two equivalents of CO₂. Intriguingly, QueD oxidation of the flavinoid quercetin results on the formation of CO, much like the off-pathway chemistry of ARD. However, only the QueD from *Streptomyces* appears to use Ni²⁺ as a co-factor.^{65–66} OxDC and OxOx are both bicupins, containing one Mn²⁺ per cupin subunit. As with ARD, the metal is present in an octahedral geometry with the two open sites occupied by non-protein ligands.^{10–11} OxOx from *B. subtilis* has been studied extensively. The active

site of the enzyme contains Mn^{2+} bound in a $\text{His}_3\text{-Glu}_1$ ligation scheme essentially identical to that observed in ARD, with two water molecules completing the octahedral coordination sphere. A structure of a substrate analog (glycolate) bound to OxOx combined with EPR data were used to provide insights into the mechanism of OxOx catalysis.⁶⁷ The reaction cycle begins with monodentate binding of oxalate via displacement of one water molecule from the Mn^{2+} center. The substrate binding allows for O_2 binding to the metal by weakening the interaction of the metal ion with the second water ligand. The bound O_2 accepts an electron from Mn^{2+} to form a Mn^{3+} superoxide metallo-radical. The reaction then proceeds as shown in Scheme 9. In the proposed mechanism, the metal not only binds both substrates (oxalate and O_2), organizing them for reaction, but also transiently reduces dioxygen to superoxide. It is important to note that this mode of glycolate binding observed in the protein crystal is distinct from that observed in small molecule inorganic complexes of Mn^{2+} and glycolate. In the small molecule model studies, glycolate exhibits bidentate coordination.⁶⁸ The protein complex may exhibit the extended, monodentate coordination due to steric barriers disfavoring bidentate coordination, with specific stabilization of monodentate ligation through steric interactions with active site residues. A similar mode of substrate binding, where oxalate binds in an end-on conformation providing an empty coordination site for dioxygen ligand was observed in oxalate decarboxylase.⁶⁹

8.1. Enzymes related to Ni-ARD

Quercetinase (QueD) is a flavonol 2, 4-dioxygenase involved in the oxidative degradation of quercetin, a common constituent of foodstuffs such as berries, tomatoes and black tea. It is a CO-releasing dioxygenase and is probably the closest enzyme to Ni-ARD in terms of the chemistry it performs (Scheme 8). While fungal quercetinases are exclusively copper dependent enzymes,^{70–74} bacterial quercetinases from *B. subtilis* and *Streptomyces* were reported to be promiscuous enzymes similar to KoARD. In *B. subtilis* QueD, Co^{2+} , Cu^{2+} , and Mn^{2+} can support catalysis but the catalytic efficiency is the highest for Mn^{2+} .^{75–76} *Streptomyces* QueD was found to be active with Ni^{2+} , Co^{2+} and Mn^{2+} but in contrast to *B. subtilis* QueD, was most active with Ni^{2+} .⁷⁷ Fe^{2+} was shown to be a poor cofactor in both the bacterial species. Although these bacterial QueDs are promiscuous, no changes in the enzymatic chemistry based on the identity metal cofactor as seen in ARD enzymes have been reported.⁷⁷ Only CO and 2-protocatechuoylphloroglucinol carboxylic acid, were detected for Co^{2+} -bound QueD in the *B. subtilis* study or for the Co^{2+} and Ni^{2+} isoforms from *Streptomyces*. Characterization of bacterial QueDs used recombinant proteins expressed in *E. coli*. As the native protein from *B. subtilis* has not been purified and characterized, the identity of the preferred metal cofactor for this QueD remains unknown. On the other hand, QueD was isolated from the wild-type *Streptomyces* sp. strain FLA and was found to contain mainly nickel and zinc.⁷⁷ Hence Ni^{2+} is most likely the physiologically relevant cofactor of QueD from *Streptomyces* sp. FLA.

The resting state and substrate/inhibitor bound structures of QueDs from *Aspergillus japonicus* and *B. subtilis* have been solved.^{70, 75, 78–79} However, the catalytic mechanism of these enzymes is still under debate: Whether O_2 directly binds the metal ion prior to reaction with substrate or reacts directly with the activated substrate is unknown. There are several reasons to believe that direct binding of dioxygen to the metal ion may not be important: As

with acireductone, quercetin can react spontaneously with O₂ in the absence of QueD, and bacterial QueDs are promiscuous, which suggests that redox activity of the metal cofactor is not essential for catalysis. Small molecule models of QueD activity do not require an open coordination site at the metal for breakdown of quercetin with dioxygen. Furthermore, cofactor-independent dioxygenases are known to catalyze the cleavage of heteroaromatic rings with dioxygen in the absence of metal cofactors.^{80–82}

Given the structural and functional similarities between Ni-KoARD and *Streptomyces* QueD, the mechanism of QueD activity is likely applicable to the details of Ni-KoARD function. Structures of *Streptomyces* Ni-QueD in the resting state and bound to substrates quercetin and O₂ have been determined by crystallographic cryotrapping approach, providing direct insight into how quercetin and O₂ are activated at the Ni²⁺ ion (Scheme 10).⁶⁵ The Ni²⁺ ion is present in an octahedral geometry coordinated by three His residues (His69, His71 and His115) and a Glu residue (Glu76) in all three states. In the resting state, the two non-protein ligands are water molecules. Upon binding, quercetin replaces one water molecule. This binding rotates the carboxylate group of Glu76 by 90°, which then forms a hydrogen bond with quercetin and stabilizes the enzyme-substrate complex. This conformational change weakens the interaction between Ni²⁺ and the second water ligand, which is then replaced by side-on (π) O₂ binding to Ni²⁺. The reaction then proceeds with cleavage of the C2–C3 and C3–C4 bonds of quercetin. This is the first direct experimental evidence for direct substrate-O₂-metal interaction in this class of enzymes. There has been no experimental evidence to date to indicate the formation of a stable M²⁺-O₂ complex in either Fe- or Ni-ARD.

8.2 Enzymes related to Fe-ARD

A comprehensive search in the MetalPDB⁸³ database for dioxygenases with Fe²⁺ with octahedral coordination geometry including Glu and His ligation finds homoprotocatechuate 2,3-dioxygenase (HPCD), 3-hydroxyanthranilate 3,4-dioxygenase (HAD) and homogentisate 1,2-dioxygenase (HGDO) as likely matches to Fe-ARD. These enzymes present an active site with Fe²⁺ present in an octahedral coordination, but through a His₂-Glu₁ motif rather than the His₃-Glu₁ found in ARD. Water molecules occupy the non-protein ligation sites in the octahedron. Crystal structures of the resting HPCD enzyme-substrate and enzyme-substrate-O₂ complexes indicate that the ferrous ion is involved in binding both the substrate and O₂ prior to reaction. The mechanism appears to require first binding of the substrate, displacing the water ligands to the iron. Substrate binding increases the affinity of the metal center for O₂ binding, with the reaction then proceeding subsequent to O₂ binding.^{84–86} Scheme 11 shows the proposed mechanism for HPCD, an example of an extradiol catechol dioxygenase.

Crystal structures show that the substrates for HAD and HPCD are both bound in bidentate fashion whereas the substrate for HGDO exhibits monodentate binding to the ferrous ion. Conversely, O₂ binds the ferrous ion side-on in HGDO and HPCD and end-on in HAD (Scheme 12).

9. Summary and Discussion

9.1 Metal-dependent function of ARD and potential roles in carcinogenesis

ARD from *Klebsiella oxytoca* (KoARD) is currently the only known enzyme that, as isolated from the native organism, performs two different chemistries based on the metal ion cofactor bound to the protein in the active site.^{1–2} The function of the off-pathway chemistry in *Klebsiella oxytoca* is unknown, it can be speculated to be involved in the regulation of the MSP. We have shown that mammalian ARDs (mouse and human) also exhibit this metal ion-dependent on- and off-pathway chemistry *in vitro* where the Fe²⁺-bound forms catalyze on-pathway and the Ni²⁺, Co²⁺ and Mn²⁺-bound forms catalyze off-pathway chemistry.^{3–4} Unlike ARD in *Klebsiella oxytoca*, where Ni-KoARD has a higher activity than Fe-KoARD³⁷, the mammalian ARDs exhibit the opposite behavior, with the Fe²⁺-bound form of the enzymes exhibiting several fold higher activity than the Ni²⁺, Co²⁺ and Mn²⁺-bound forms. In all cases tested to date, the Ni²⁺-bound ARDs are the most thermostable, followed by Co²⁺, Fe²⁺ and Mn²⁺ respectively. A eukaryotic ARD from *Oryza sativa* L. (OsARD) has also been studied biochemically and the Ni²⁺-bound OsARD (which exhibits the off-chemistry) polymerizes and has a much reduced activity relative to Fe²⁺-bound OsARD (exhibits on-pathway chemistry).⁸

Since Fe²⁺-bound mammalian enzymes exhibit the maximum on-pathway enzymatic activity and many oxidoreductases use Fe²⁺ as a metal ion co-factor,⁸⁷ we suspect that the Fe²⁺-bound form is the native form of ARD under normal conditions in mammals. Immunoprecipitation experiments to isolate native ARD from mice liver can be used to identify the native metal cofactor. The reduced activity of Ni²⁺-bound mammalian and eukaryotic ARD enzymes suggests that off-pathway chemistry catalyzed by the Ni²⁺-bound enzyme may not be relevant in a healthy organism and may be only seen only in pathology. However, given the increased thermal stability of the Ni²⁺-bound enzyme relative to the Fe²⁺-bound form, formation of Ni-ARD could represent a pathological kinetic trap *in vivo* in mammalian systems. Given the very low expected concentrations of nickel *in vivo* (< 2 µg/L in serum or other biological fluids)⁸⁸, and the known carcinogenicity of soluble nickel compounds,⁸⁹ it is possible that the formation of a stable Ni-ARD may be involved in tumor development and/or other pathological conditions in humans and other mammals.

This is an especially intriguing possibility due to the known up- and down-regulation of the *ADII* gene in multiple types of carcinoma.^{15, 56–58} Multiple independent studies have indicated a role for HsARD in cancer. Seiki, *et al.* have shown that HsARD binds the cytoplasmic tail of MMP14 and inhibits cell migration, metastasis and tissue invasion.¹⁶ Oram *et al.*, have shown that *ADII* is down-regulated in rat and human prostate cancer cell lines and the enforced expression of *ADII* causes apoptosis.¹⁵ Besides the role of HsARD in regulating the activity of MMP, the metal-dependent dual-chemistry of mammalian ARD enzymes is particularly interesting in that carbon monoxide (CO) which is a product of the off-pathway chemistry, is known to be an anti-apoptotic molecule and has been proposed to act as a signaling molecule analogous to NO.^{13, 90–92} This leads to the intriguing possibility that binding of Ni²⁺, Co²⁺ or Mn²⁺ in the ARD active site could lead to inappropriate gain-of-function in carcinogenesis, helping to protect transformed cells from apoptosis by

producing CO and/or loss-of-function in regulating MMP14 activity. Experiments with rat liver homogenates indicated presence of only on-pathway ARD activity, with CO formation not detected.²

9.2 Implications for the mechanism of ARD-catalyzed dioxygenation reactions

The Abeles and Pochapsky groups have comprehensively characterized the dual chemistry of KoARD in terms of mechanistic enzymology.^{1, 37, 48} Kinetic analyses have indicated that both Ni-KoARD and Fe-KoARD follow a sequential mechanism, i.e. both substrates must bind before the products are released. UV-visible spectroscopy indicates that acireductone substrate binds to both Fe-KoARD and Ni-KoARD (ES complex formation) regardless of whether oxygen is present. Oxygen does not bind to either of the enzymes in the absence of substrate, but reaction is rapid enough once added to the ES complex that binding of oxygen by the metal (if it is occurring) cannot be easily measured. Titration studies of the model substrate used in these experiments indicated that its maximum absorption depends on its ionization state. The pK_a values of the model substrate are 4.0 and 12.2 and its λ_{max} shifts from 305 nm in singly deprotonated form (pH 7) to 345 nm as a dianion. Although the authors suggested that acireductone binds as a dianion to KoARD (in both forms) based on spectroscopic shifts, they were unable to see the formation of a complex between Ni^{2+} or Fe^{2+} and acireductone di-anion in solution spectroscopically at pH 13.0.³⁷ Hence the precise nature of the interaction of the substrate with enzyme can only be determined by high-resolution structural studies. However, NMR and XAS experiments on KoARD showed that acireductone directly ligates the metal center in the ES complex.⁹³

Several hypotheses for the dual chemistry of KoARD have been proposed. The chelate hypothesis postulates that the difference in the chemistry is due to a difference in the coordination modes of substrate to the metal ion center, where Fe^{2+} forms a five-membered ring and Ni^{2+} forms a six-membered ring leading to different products.^{20–21, 37} Small molecule model studies and computational studies challenged the chelate hypothesis, showing that both Ni and Fe can form six-membered ring.^{18–19, 63, 94} However, the D-LA-bound Ni-MmARD structure demonstrates that five-membered rings can indeed form involving the Ni^{2+} ion and a substrate analog. Furthermore, although several potential ligands were tested, no six-membered bidentate metal-ligand complexes were observed in our MmARD crystallographic structures. While not conclusive evidence for a five-membered ring intermediate in ARD catalysis, these results suggest that the mechanism is still an open question. What can be concluded from the current state of knowledge regarding the mechanism of ARD catalysis is that binding of the acireductone substrate is the first step in catalysis. However, how (or even if) O_2 binding to the metal is required for activation by ARD is still not known. None of the proposed mechanisms require an assumption of metal-oxygen binding, although they all presume a bidentate ES complex. Although reaction of O_2 with singlet compounds typically requires activation of the ground-state triplet dioxygen, the acireductone functionality (as typified by ascorbic acid) is a good reducing agent, and some triplet character may be generated on the bound acireductone via interaction with the paramagnetic metal ion with which it is complexed in the ARD active site, making a single electron transfer to O_2 to generate a radical cation anion pair less forbidden.^{20–21, 37} For the most part, mononuclear non-heme Fe^{2+} -containing-dioxygenases (e.g., extradiol catechol

dioxygenases) require direct O₂ binding to Fe²⁺ in the for activation. However, Fe³⁺-containing dioxygenases (intradiol catechol dioxygenases) can activate the substrate, rather than dioxygen, in order to facilitate reaction.⁹⁵

Unlike ARD, the structure of Ni²⁺-bound *Streptomyces* QueD ES-O₂ complex does indicate a direct metal-O₂ interaction.⁶⁵ The structural data suggests that before the reaction proceeds, Ni²⁺ activates both the substrate and dioxygen through direct binding, although the electronic state of the ternary complex remains unresolved. The authors speculate the bound O₂ to be a superoxide species based on the O-O bond length. The authors thus propose that the reaction mechanism of *Streptomyces* Ni-QueD is similar to that of Fe²⁺-dependent dioxygenases (extradiol catechol dioxygenases) such as HPCD. The presence of Ni²⁺ to activate O₂ is unexpected since Ni²⁺ does not typically have accessible redox chemistry under physiological conditions. Similar to the mechanistic proposition for HPCD, it is likely that one or two electrons may pass from the Ni²⁺-bound substrate to the O₂ with either no change in the oxidation state of Ni or a transient change that persists for much less than the freezing time for rapid freeze quench (RFQ) crystallography experiments or EPR experiments.⁸⁶ Although there are several similarities between *Streptomyces* Ni-QueD and Ni-MmARD, the structure of the ES complex in Ni-QueD shows monodentate binding to the Ni²⁺ ion, displacing a solvent ligand. The other solvent ligand is replaced upon dioxygen binding to Ni²⁺. There is no spectroscopic data to support one mode of substrate binding (monodentate or bidentate) over the other in Ni-MmARD. However, the structure of D-lactic acid bound to Ni-MmARD suggests that bidentate binding is possible. If the substrate binds in a bidentate fashion, there is no open coordination site for dioxygen binding unless a protein ligand dissociates from the metal. Spectroscopic studies on KoARD suggested bidentate binding of the substrate, but the authors state that monodentate binding cannot be ruled out.³⁷ Furthermore, EXAFS data of the resting state and ES complex of KoARD suggests that one His could be displaced by substrate binding,⁹³ leaving room for dioxygen binding, even if the substrate remains bound in bidentate fashion.

A structure of the ternary ES-O₂ complex would be extremely useful for obtaining mechanistic details for Ni-MmARD. However, given the sensitivity of acireductone to oxidation, it is unlikely that such a structure will be obtained with current methods. The structure of Ni-MmARD bound to an oxidatively stable competitive inhibitor might provide details on the substrate binding modes, including the possibility of O₂ binding. Another important advance would be to crystallize an Fe²⁺-bound ARD isoform. The Fe²⁺-bound MmARD is oxidatively unstable and requires anaerobic handling due to oxidation from the Fe²⁺ to Fe³⁺ state. The Fe³⁺ form of the enzyme is unstable and exhibits a tendency to autoproteolysis. Although Fe-MmARD was purified and set for crystallization anaerobically, repeated attempts to crystallize it have failed. However, multidimensional NMR spectra of Fe-HsARD have been recently obtained and analyzed, and sequential resonances assigned. Preliminary results suggest that, as with Fe-KoARD, the C-terminal peptide of Fe-HsARD is disordered, and significant structural differences exist between the Fe- and Ni-bound forms of HsARD (Figure 10).⁴

The 1D and 2D NMR spectra of metal isoforms of HsARD in Figure 10 shows that, although the signals from Mn²⁺-bound HsARD (pink in both spectra) are quite broad due to

paramagnetic relaxation, they are virtually superimposable on the corresponding signals from Ni-HsARD (in green). This suggests that the structures of the Mn^{2+} and Ni^{2+} HsARD isozymes are very similar. On the other hand, the octahedral complexes of Fe^{2+} and Ni^{2+} with N/O ligation both have integer electronic spins ($S=2$ and $S=1$, respectively) and have similar ^1H pseudocontact shift patterns and magnitudes in model complexes.⁹⁶ As such, the spectral differences between the Fe^{2+} and Ni^{2+} HsARD isoforms likely reflect genuine structural differences, highly reminiscent of what was observed in KoARD.²⁰ The Co^{2+} -HsARD ($S=3/2$) probably exhibits significantly different pseudocontact ^1H shifts than the Ni^{2+} -bound enzyme, again based on the results from the model complexes. So, while the similarity between the crystallographic structures of Ni-MmARD and Co-MmARD (RMSD = 0.06 Å) may be to some extent crystallographically artifactual, resolving this issue must await the results of a more complete NMR structural characterization of these HsARD isoforms.

9.3 Moonlighting function(s) of HsARD

ARD has been shown to serve regulatory functions in mammals in addition to its enzymatic function in the MSP. HsARD binds the cytoplasmic tail of membrane-type 1 matrix metalloproteinase (MT1-MMP) and acts as a negative regulator by inhibiting MT1-MMP-mediated cellular invasiveness.¹⁶ *ADII* encoding ARD is downregulated in rat prostate and human prostate cancer cell lines, cultured gastric carcinoma cells, fibrosarcoma cells and glioblastoma cells. Enforced ARD expression induces apoptosis.¹⁵ *ADII* gene expression has been identified as having a direct link with carcinogenesis in a clinical study testing the impact of environmental carcinogens on gene expression differences in humans.⁵⁸ Yeh, *et al.* have identified an N-terminal truncated version of HsARD which is implicated in the replication of hepatitis C virus in non-permissive cell lines.⁵ The MT1-MMP and *ADII* interaction suppresses HCV infection and this inhibition can be reversed by *ADII* overexpression.⁵⁹ *ADII* is also required for normal *Drosophila* fecundity.⁷ *ADII* expression is impacted in Down's syndrome (DS) and DS-associated congenital heart defects (DS-CHD) with higher expression seen in fetuses trisomic for Hsa21 than in normal control fetuses. These multiple roles for ARD in human physiology and pathology leads to interesting questions: Do each of these functions have a different mechanism or are they inter-related? Are these functions performed in different subcellular compartments? Are these non-enzymatic functions dependent on the enzymatic activity of ARD? Future work can be addressed towards delineating some of these functions of ARD and identifying regions of the protein that are involved in non-enzymatic functions of ARD.

10. Perspective

10.1 Challenges in working with promiscuous metalloproteins

The biochemical and mechanistic study of metalloproteins can be complicated due to several reasons, including difficulty in obtaining a homogeneous purified sample with only a single metal bound, difficulties in identification of the native metal. This becomes even more challenging in the case of a promiscuous binder such as ARD, in which any metal impurity at any step of expression, isolation or purification can complicate analysis. Having met this

challenge in the case of ARD expression, purification and characterization, we will summarize our findings for the edification of interested researchers.

10.2 Isolation of metalloprotein samples with a single metal bound

Biochemical and structural characterization of metalloproteins can be both unsatisfying and ambiguous if the sample is a mixture of several metal bound forms (and, if the metals are redox active, this ambiguity can be further exacerbated by mixed oxidation states!). The incorporation of a single metal ion in a recombinantly expressed metalloprotein can be experimentally challenging. We have used several approaches to obtain a single-metal bound form of ARD enzymes. These include preparation of the apoprotein and reconstitution with the desired metal⁴⁸ and expression of the protein in minimal media in the presence of excess of a single metal.³⁻⁴ In order for the first method to work, the apoprotein must be either stably folded in the absence of metal or refolded in high yield upon addition of metal. In the case of ARD, we have found that while the bacterial enzyme can be refolded and repurified, yields tend to be low. Expression of the protein in media enriched with the desired metal has allowed us to obtain sufficient quantities of ARD highly enriched in a single metal for all of our experimental needs, including crystallography and NMR.³

Another issue that can hamper characterization of metalloproteins is the common use of metal affinity tags for purification. The His tag used routinely for purification of overexpressed proteins is not recommended for metalloproteins, for obvious reasons. The affinity of the tag for metal ions such as Ni²⁺ and Co²⁺ can complicate metal analysis and, in the case of the ARD enzyme family, introduce uncertainty regarding the reasons for product distributions. We have found that the Strep tag (WSHPQFEK) tag with affinity for streptavidin provides a high degree affinity purification for ARD enzymes.³ Affinity purification minimizes the number of purification steps, which can be critical if the metal is relatively weakly bound to the protein and might be lost during purification. This is particularly useful for the purification of Fe²⁺-bound ARD enzymes, as oxidative loss of ferrous iron is a serious issue. In all cases, we test the recombinant metalloprotein for metal content using ICP mass spectroscopy to determine the homogeneity of the sample. Furthermore, anomalous scattering measurements provide unambiguous information regarding metal identity in crystalline enzymes. Since each metal has a distinct anomalous scattering wavelength, X-ray diffraction data collected slightly below and above the absorption edge can be used for unambiguous metal identification. To confirm the identity of the active site metals as Ni or Co in MmARD, X-ray datasets were collected 100 eV below and above the absorption peaks of the K-absorption edges of each of these metals.³ Strong peaks were observed in the expected metal position in the anomalous electron density maps calculated using the above absorption edge X-ray data whereas much weaker peaks were observed in the corresponding position calculated using the below absorption edge X-ray data. This sharp change in anomalous scattering on passing through the absorption edges of Ni or Co confirmed the metal identity for each case of MmARD structure determinations.

10.3 What is the *in vivo* metal cofactor of my metalloprotein?

Determination of the physiologically relevant metal ion of a metalloprotein can be a challenging task. This is especially true when the metal is loosely bound, or (as is often the

case) the protein is expressed heterologously. There are several examples of proteins whose native metal identity is still under debate (examples discussed below). Purification of the native enzyme from the wild-type organism and conducting metal analysis on the purified protein is the ideal method to establish metal identity, but this is not always possible due to low yields. Recombinant heterologous expression is the most common method of obtaining proteins for biochemical studies.

There are approaches that can help in the identification of the *in vivo* metal cofactor, even in the absence of isolation of the native enzyme. Comparison of biochemical properties such as activity and stability profiles of the native enzyme (in fresh cell lysates) with those of purified recombinant proteins bound to various single metal ions can provide an idea of the likely native metal cofactor. Metal abundance in the native organism and metal coordination preference is also an important factor to consider in such studies. However, this is not necessarily definitive, given that metal sequestration and delivery is often complex and highly evolved. For example, ferrous iron was shown to be the likely native metal of the enzyme peptide deformylase (PDF). PDF catalyzes the removal of N-terminal formyl groups from nascent ribosome-synthesized polypeptides. It was proposed that PDF from *E. coli* is Fe²⁺-dependent (Fe-EcPDF) since the activity and stability (oxidative instability) of recombinant Fe-EcPDF were similar to those of native EcPDF.⁹⁷ But the assumption that this was true for all bacterial PDFs was shown to be incorrect by Nguyen *et al.* who studied PDF in *Borrelia burgdorferi* (BbPDF) and *Lactobacillus plantarum* (LpPDF) which grow in Fe-limited conditions.⁹⁸ In their study, they showed that purified native BbPDF contains approximately one mol Zn²⁺/mol protein and no detectable levels of iron, and that recombinant BbPDF when expressed in medium containing both zinc and iron, selectively binds zinc, while EcPDF preferentially binds Fe²⁺ when overexpressed in rich medium and binds Zn²⁺ only in iron depleted medium. A number of other Fe²⁺-dependent enzymes such as methionyl aminopeptidase⁹⁹, *S*-ribosylhomocysteinase (LuxS)¹⁰⁰, UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC)¹⁰¹, and histone deacetylase-8 (HDAC8)¹⁰², γ -carbonic anhydrase¹⁰³, cytosine deaminase¹⁰⁴, and atrazine chlorohydrolase¹⁰⁵ were initially misidentified as Zn²⁺ metalloenzymes because they were purified under aerobic conditions leading to oxidation of Fe²⁺ to Fe³⁺ followed by dissociation of Fe³⁺ and replacement with Zn²⁺.

The identity of the *in vivo* metal cofactor is clearly important for developing effective therapeutic inhibitors. Most inhibitors of metalloenzymes bind to the catalytic metal ion and affect not only the catalytic activity but also substrate affinities. Consequently, the identity of the native physiological metal cofactor is essential for the design of effective and specific inhibitors. One such example is histone deacetylase 8 (HDAC8), which has been validated as a cancer target.¹⁰⁶ Human HDAC8 retains catalytic activity with a number of divalent metal ions in the order of specific activity: Co²⁺ > Fe²⁺ > Zn²⁺ > Ni²⁺. It is postulated that Fe²⁺ may be the native metal in human HDAC due to the oxidative instability exhibited by this enzyme. The affinity of the HDAC8 inhibitor suberoylanilide hydroxamic acid (SAHA) and the enzyme kinetic parameters are influenced by the metal ion identity with 5-fold lower K_M value for Fe²⁺-bound HDAC8 compared to Zn²⁺-bound HDAC8.¹⁰²

We expect that this question will be critical if HsARD is to be targeted for any disease state functions. If Fe^{2+} is the co-factor under normal physiological conditions (an assumption that seems reasonable but is not yet proven), what metal(s) are present when the enzyme is expressed in tumors or in disease tissue? Is the off-pathway chemistry observed in these tissues, or is the role of HsARD in these states not a function of the metal, but unrelated? These questions are currently the subjects of our investigations.

10.4. Metal-dependent chemistry in metalloproteins

The various structural genomics initiatives have produced structures of many metalloproteins, but the identity of the metal ion in these structures are often ambiguous. In fact, given that heterologous expression systems combined with affinity labels such as His tags are ubiquitously used for protein preparations in high-throughput projects, this is a serious issue. As we and others have seen with ARD, the metal ion not only determines the enzymology but can have strong influence on structure as well. There have been several examples in the literature where authors have studied the impact of different metals on enzyme kinetics of a metalloprotein.^{98, 100, 102, 107} It is often assumed that a metalloenzyme will perform the same chemistry irrespective of the metal in the active site, and changing the metal will only change the kinetic parameters of the enzyme, but not the product(s). The dual chemistry exhibited by ARD clearly shows that this assumption can be misleading. It is critical that future metalloenzyme research tests the effect of different metals not only on enzyme kinetics but product distributions as well.

10.5. Metal sensing in biological systems

Metal sensing and metal homeostasis is a critical function for all organisms. Metal ions are ubiquitous in the environment, and many are toxic if present in the cytosol in high concentration, or injurious if not present in sufficient amounts. It is no surprise then that extensive metal sensing and transport systems have evolved in order to remove toxic metals or take in and appropriately compartmentalize required metals.^{108–109} Still, relatively little is known regarding the mechanisms by which one metal ion is distinguished from another, especially in cases where ions have the same charge and similar sizes. Although many metal-containing co-factors (e.g., heme, chlorophyll, cobalamin) are pre-formed when finally bound to their protein partners, this still requires that the co-factors themselves be properly synthesized with the correct metal. Alkali metal and alkaline earth ions are sometimes bound via amide carbonyl oxygens (as in selective ion channels¹¹⁰ or when stabilizing local structural motifs¹¹¹). In these cases, the protein backbone conformations appear to be pre-arranged to selectively bind cations of the appropriate size and charge. Serine and tyrosine hydroxyl groups are often involved in Ca^{2+} binding, which triggers major conformational changes in calmodulin.^{112–113} However, the vast majority of proteins incorporating transition metals use a common set of ligands: histidine (imidazole), aspartate and glutamate (carboxylate) and cysteine or methionine (thiolate and thioether, respectively). Within this group, histidine can ligate through either imidazole nitrogen, but due to steric considerations, Nε ligation is the most common. Carboxylates also have the possibility of mono- or bidentate ligation, with the increased flexibility of the glutamate side chain relative to aspartate providing a variety of steric possibilities for metal-ligand interactions. Depending upon identity and oxidation state, many transition metals have a preferred

coordination geometry that pre-disposes certain ligand arrangements within a polypeptide to bind a particular metal selectively. However, this is not the only determinant, as we have found that Zn^{2+} does not form a stable monomeric complex with KoARD, while the *Streptomyces* QueD can incorporate zinc.^{20, 77} Fierke and co-workers found that Zn^{2+} can replace Fe^{2+} in a bacterial deacetylase (LpxC) depending upon cellular conditions, and that both are active enzymes, although ferrous ion is bound more tightly than zinc and results in a more efficient enzyme.^{101, 114}

The response of a protein to the binding of a metal can vary from the protein being disordered in the absence of the metal (e.g., zinc finger motifs and many ferredoxins¹¹⁵) to a more general “tightening” of the structure, as seen in some sensor proteins and globins.^{116–117} This “tightening” is usually reflected in increased denaturation temperatures and decreased amide H/D exchange rates, and/or decreases in hydrodynamic radii.^{111, 116} Metal binding at an interface between subunits can modulate affinity for second-site metal and interaction partners in activators, promoters and inhibitors of transcription.^{118–119} In the case of KoARD, the apo-enzyme appears to fold in much the same manner as the Fe^{2+} -bound enzyme, suggesting that the “default” fold of KoARD is pre-disposed to bind iron without significant structural rearrangement. This is inferred from the observation that the metal-free mutant H98S KoARD is stably folded and does not exhibit significantly more dynamic line broadening in NMR spectra than the WT enzyme with metals bound.²⁰

What renders the metal sensitivity of the ARD enzymes unusual is the fact that the off-pathway (Ni^{2+} -bound) isoforms of all of the ARD enzymes characterized to date exhibit significantly higher thermal stability than any other metal-bound form, including the presumed “default” Fe^{2+} -bound isoforms. In the case of KoARD, we have proposed that the fixed arrangement of three of the four protein-based ligands (His96, His140 and Glu102) within the cupin barrel combined with Ni-N/O bonds on average 0.1 Å shorter than the corresponding Fe-N/O bonds pulls the fourth ligand (His98) deeper into the active site. This allows the formation of stable hydrogen bonds not present in the iron isoform, and dramatically increases the ordering of the polypeptide around the active site. In turn, this ordering triggers a substantial re-arrangement of a hydrophobic core region and rearrangement of helices on the distal face of the cupin barrel, as well as the formation of a C-terminal helix on the distal face. Conversely, a region that is relatively well ordered in the Fe^{2+} -bound KoARD (the D loop) is disordered in the Ni^{2+} -bound enzyme. Based upon spectral evidence, this is also likely to be true for HsARD as well, suggesting that the metal-dependent structural switching is evolutionarily conserved.

10.6 Synthetic biology and artificial metalloproteins

The field of artificial metalloenzymes has expanded significantly over the last decade to explore some of the unaddressed challenges in synthetic organic chemistry. Transition metal complexes catalyze a broad range of chemical reactions used to prepare industrially important chemicals.¹²⁰ In general, transition metal catalysts provide only the first coordination sphere to modulate reactivity. Metalloenzymes, on the other hand, can modulate both the first and second coordination spheres to provide substrate specificity and product selectivity. This makes modulation of native metalloenzyme function to provide

useful high-value products an attractive goal. There are several different approaches in creating artificial metalloenzymes, including introduction of a novel function through site specific modification and directed evolution,^{121–122} introduction of an organometallic catalyst into a scaffold protein, and introduction of a novel function by replacement of the native metal with an abiotic metal.¹²³

Enzymes have evolved to achieve the catalytic efficiency and selectivity required for controlled biochemical processes. It is becoming clear that not just the active site, but the entire enzyme structure is involved in this control.¹²⁴ As such, re-engineering to improve or modify an enzyme to reach high efficiencies must take this requirement into account. The most straightforward way of mimicking natural selection is to use directed evolution, with iterative rounds of enzyme diversification and functional screening. Cytochrome P450s have been modified and evolved using directed evolution protocols to catalyze a wide range of non-natural reactions.¹²⁵ Similarly, Kim and coworkers conferred β -lactamase activity to glyoxalase II using directed evolution.¹²⁶ Seelig and Szostak found that randomization of two loops found in the zinc-finger domain of a retinoid-X-receptor protein induced RNA ligase activity.¹²⁷ Hayashi and coworkers engineered myoglobin to obtain peroxidase activity by introducing site specific mutations and replacing the porphyrin ring with a “double winged” cofactor (replacing the propionate groups of the heme with nonpolar aromatic moieties).¹²⁸ The normally non-catalytic myoglobin has also been engineered to perform carbene insertion into N-H bonds,¹²⁹ alkenes¹³⁰ and S-H bonds¹³¹ and nitrene transfer reactions to effect C-H amination¹³² by introducing a substrate binding cleft through site specific mutations.

Another approach is to incorporate an organometallic catalyst into a protein scaffold to create artificial metalloenzymes. This, it is hoped, will combine the substrate selectivity of enzymes with the efficiency of homogeneous transition-metal catalysts. Pioneered by Wilson and Whitesides,¹³³ the streptavidin-biotin system has been researched extensively for non-covalent anchoring of biotinylated organometallic moieties within streptavidin to form artificial metalloenzymes. Several reviews have described this system, which has been used for a variety of enantioselective transformations including hydrogenation, trans-olefination, metathesis, allylic alkylation and sulfoxidation.^{125, 134–138} Replacement of the native organometallic cofactor with an abiotic cofactor has also been used as a strategy to introduce novel function in heme proteins because of the ability of the porphyrin to accommodate metals other than iron, or, alternatively, bind other polycyclic metal co-factors into the heme binding pocket. The porphyrin ring in myoglobin has been substituted with a variety of artificial cofactors, including substituted porphyrins such as porphycene^{132, 139} metal-Schiff base complexes,^{140–142} metallo-salophen,^{140, 142} metallo-salen^{141, 143} and Rh phebox¹⁴⁴ complexes. These substituted Mb-hybrid systems have activities exhibited by the synthetic metal catalysts. A variety of other protein hosts have also been used for covalent anchoring of artificial metallocofactors which include albumins,^{145–148} proteases,^{149–153} lipases,^{121, 154–155} anhydrases^{156–159} and other proteins.^{160–161} Organometallic cofactors have also been introduced into cage proteins, providing an interior cavity for immobilization of the catalyst. Ferritin has been shown a good candidate for immobilizing a range of metal catalysts and serving as a catalytic reaction space.^{162–165}

A third approach, replacement of the native metal with an abiological metal is relatively unexplored. ARD is the only example of a natural metalloprotein which exhibits completely different chemistries when bound to different metals. Replacement of the native metal with an abiotic metal is attractive in that it is a simple approach and can be used to explore novel chemistries not typically seen in enzymes. There are a few examples in literature describing this approach. The Zn²⁺-binding carboxypeptidase A (CPA) was substituted by Kaiser and coworkers with Cu²⁺, converting it into an oxidase. This Cu-CPA oxidase catalyzes the oxidation of ascorbic acid to dehydroascorbic acid, but lacks the peptidase and esterase activities of the native enzyme.¹⁶⁶ In another case, Zn²⁺ ion of human carbonic anhydrase (hCA) was removed and replaced with Mn²⁺ and Rh²⁺. hCA replaced with Mn²⁺ catalyzed an enantioselective artificial epoxidase¹⁶⁷ while hCA replaced with Rh²⁺ catalyzed hydrogenation and regioselective hydro- formylation reactions.^{168–169} A recent study has reported replacement of iron in Fe-porphyrin proteins with abiological noble metals to create enzymes that catalyze reactions not catalyzed by native Fe-enzymes or other metalloenzymes.²² Artificial metalloenzymes obtained by replacement of the native metal with an abiotic or non-native metal can serve as a unique opportunity for protein engineering to expand the repertoire of reactions catalyzed by native metalloenzymes.

10.7 Conclusions

It has been over twenty years since ARD was first discovered in bacteria^{2, 35–36}, and a little over ten years since it was discerned that humans and other eukaryotes encode a similar enzyme. It is worth noting that the motivation leading to ARD's discovery was the observation that many human cancer cell lines have a methionine dependence, and identifying the origins of that dependence could lead to potential therapies.¹⁷⁰ In retrospect, it is intriguing that the *ADII* gene product was first identified not as an enzyme, but as a regulator of matrix metalloproteinase activity, viral infection and apoptosis. The context-dependent nature of those regulatory functions was (and remains) puzzling, but in light of the recent confirmation that HsARD also exhibits the dual chemistries of the bacterial enzyme, there is some hope that the context dependence of other, non-enzymatic, functions of ARD might be related to the structural and chemical changes brought by binding different metals in the ARD active site.

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Biographies

Aditi Deshpande received her Bachelors in Chemical Engineering from Institute of Chemical Technology, India and Masters in Chemical Engineering from University of Delaware. She worked for 5 years in the biotech industry in process development and manufacturing of biologics. In 2010, she joined Dagmar Ringe and Gregory Petsko's research group at the Brandeis University where she investigated the metal-dependent behavior of mammalian acireductone dioxygenase enzymes. She received her Ph.D. in biochemistry & biophysics in 2016 from Brandeis University and is currently working as a scientist at Allena Pharmaceuticals. Her research interests include enzymology, protein structure, biocatalysis and design of artificial metalloenzymes.

Thomas was born in 1954 in Cleveland, Ohio. He received his B.Sc. in analytical chemistry from the University of Pittsburgh in 1977, and worked for several years in industry before joining William Pirkle's research group at the University of Illinois. While there, he invented a series of novel chiral stationary phases and provided spectroscopic evidence to support their mechanism of enantioselectivity. He received his Ph.D. in organic chemistry in 1986, and did post-doctoral research with Stephen Sligar at Illinois and Peter Wright at Scripps. He began his career as assistant professor of organic chemistry at Brandeis University in 1989. He is currently Professor of Chemistry and Biochemistry at Brandeis. His research

interests are in enzyme structure and function, macromolecular recognition and the molecular basis of Parkinson's disease.

Dr. Ringe received her B.A. degree in Chemistry from Barnard College in 1963 and Ph.D. degree in Organic Chemistry from Boston University in 1968. She is currently the Harold and Bernice Davis Professor of Aging and Neurodegenerative Diseases at Brandeis University, and Adjunct Professor Neurology at Harvard Medical School. Her research is aimed at the study of the molecular mechanisms of enzymes and the functions of proteins, with a focus on the determination of function for proteins of unknown function and the development of methods for intervention on that function. The tools being used include X-ray crystallography, molecular biology, kinetics, organic synthesis, genetics, and computational approaches.

There are a number of problems to which these methods are being applied, and they include: the structural basis for efficient enzyme catalysis of proton and hydride transfer; the role of the metal ions in bridged bimetallo-enzyme active sites; direct visualization of proteins in action by time-resolved protein crystallography; the structural basis for reaction selectivity in enzymes; the evolution of new enzyme activities from old ones and the development of new pathways. The most important of these problems is the study of neurodegenerative diseases, especially Parkinson's, Alzheimer's and Lou Gehrig's diseases, with a view toward determining the functions of gene products associated with such diseases, finding common pathways that underlie them, and the search for and design of specific inhibitors or activators as potential drugs to treat these diseases. A number of methods are being used, from development of genetic and *in vitro* screens to structure-based drug design methods.

Figure 1. β -barrel structures in cupins

a) Oxalate oxidase (PDB ID: 2ET1), b) Clavaminic acid synthase (PDB ID: 1GVG) is an example of a ketoglutarate-dependent oxidase, c) homoprotocatechuate 2,3-dioxygenase (PDB ID: 3OJT) a non-heme iron-dependent dioxygenase, d) Oxalate decarboxylase (PDB ID: 1L3J). The beta sheets are colored red and the helices are blue.

Figure 2. The Methionine Salvage Pathway in *Klebsiella oxytoca*

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Figure 3. Structures of Fe- and Ni-bound KoARD

Secondary structural features are labeled corresponding to primary sequence: A: Ala2-Phe6, B: Ser14-Ser18, C: Ala21-Lys31, D: Val33-Thr49, E: Ala50-Ala60, F: Ile61-Gly69, G: Ser72-Ile76, H: Pro83-Asn94, I: Glu102-Glu108, J: Leu112-His116, K: Val121-Leu125, L: Leu131-Val134, M: His140-Met144, N: Thr151-Phe156, O: Asn158-Gly161, P: Thr162-Ile163, Q: Ile171-Tyr175.

Figure 4. Active sites of Ni- and Fe-bound KoARD

Close-up views of the active sites of Ni-KoARD (1ZRR, top) and Fe-KoARD (2HJI, bottom). Secondary structures O and P in the Ni enzyme are disordered in the Fe enzyme and are not shown in the bottom panel. The Ni view is scaled slightly smaller than the Fe view so that relevant features can be displayed.

Figure 5. Crystal structures of ARD enzymes from *B. anthracis*, *H. sapiens* and *M. musculus*
Coordinated metal ions are shown as red spheres. a) BaARD (PDB ID: 4QGM), b) HsARD (PDB ID: 4QGN), c) MmARD (PDB ID: 1VR3)

Figure 6. X-ray crystal structure of Ni-MmARD (PDB ID: 5I91)

a) The crystallographic structure of Ni-MmARD. The Nickel atom is shown as a green sphere and its protein ligands His88, His90, His133 and Glu94 represented as sticks and two water molecules are shown as red dots. b) Stereo view of nickel binding in the MmARD active site. Glu94 and His88 provide the axial ligands, with His90, His133 and two water/hydroxide ligands occupying equatorial positions, yielding an octahedral coordination geometry. The metal-ligand distances (in Å) are shown as red dotted lines. (Reproduced with permission from Ref 3. Copyright 2016 American Chemical Society).

Figure 7. The active site of Ni-MmARD showing substrate-analog and product binding
 The Ni atom is shown as a green sphere, the ligands and active site residues are shown as sticks and waters/hydroxides are shown as small red spheres. Hydrogen bonding distances are shown (in Å) as red dotted lines. Hydrophobic residues in the active site are shown in yellow. a) Stereo view of KMTB bound to Ni-MmARD. KMTB is within hydrogen bonding distance of Arg96 and the two water molecules ligated to Ni. The residues Phe84, Phe105, Phe135, Ala145, Val143 and Ile98 interact with the alkyl group of KMTB. b) Stereo view of D-Lactic acid (D-LA) bound to Ni-MmARD. D-LA replaces both equatorial water ligands, coordinating in a bidentate manner with Ni²⁺ via the carboxylate and hydroxyl oxygens. D-LA is within hydrogen bonding distance of Arg96. Residues Phe84, Phe105 and Phe135

interact with the alkyl group of D-LA. (Adapted from Ref 3. Copyright 2016 American Chemical Society).

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Saccharomyces cerevisiae -----MVKVYIHDN-KVDSYRAPHNSGTLSLDELAKLGVYKYC-----AN-
Arabidopsis thaliana  MGEAAKDQTEEVIAWYLDNKEEDQKLPHHKDPKEFVSLDKLAEGLVLCWRLDADNYET-
Mus musculus          -----MVQAWYMDESTADPRKPHRAQPRPVSLQLRTLGVLYWKLADKYEN-
Homo sapiens          -----MVQAWYMDAPGDPQPRPDPGRPVGLEQLRRLGVLYWKLADKYEN-
Klebsiella oxytoca    -----MSALTIFSVKDPQ--NSLWHSSTNAEEIQQLNAKGVRFERWQADRDLGA
Bacillus anthracis    -----ARDMAQIRIHEVNT-----RIENEVKSKFLQEGVLYEKWNISKLPFH

          :      .      .      *      **

Saccharomyces cerevisiae -----EEEVNEIARQREYKNRDVVNICESGFSKSEAEFNEKLATFYQ
Arabidopsis thaliana  -----DEELKRIRSRGYSYMDLCEVCPEKL---PNYEEKVKMFEE
Mus musculus          -----DPELEKIRKMRNYSWMDIITICKDTL---PNYEEKIKMFEE
Homo sapiens          -----DPELEKIRRNYSWMDIITICKDKL---PNYEEKIKMFEE
Klebsiella oxytoca    -----APTAETVIAAYQHAIDKLVAEKGYQSWDVISLRAD---NPQKEALREKFLN
Bacillus anthracis    LNEYSLTDENKAEILAVFSKEIADVSAARRGYKAHDVISLSNS---TPNLDELLINFQK

          . : : : * : * : : : : * :

Saccharomyces cerevisiae EHLHEDEIRYCLEGAGYFDVRDASTPENWIRCLVESGDLILPPGIYHRTLTTSNHIK
Arabidopsis thaliana  EHLHIDEIRYCLAGSGYFDVRDL--NDIWRVWVKGGILVFPAGIYHRTVDSDNMYK
Mus musculus          EHLHLDEIRYILEGSGYFDVRDK--EDKWIRISMEKGMITLPAGIYHRTLDEKNYVK
Homo sapiens          EHLHLDEIRYILDGSGYFDVRDK--EDQWIRIFMEKGMVTLPAGIYHRTVDEKNYTK
Klebsiella oxytoca    EHTHGEDEVRFVEGAGLFLHIG--DE-VFQVLCENDLISVPAHTPHWFDMGSEPNFT
Bacillus anthracis    EHHHTDDEVRFIVSGHGIFAIEGK--DGTFFDVELEPGDLISVPENARHYFTLQDDRQVV

** * :*:*: : * * * :. : : :. * * * :.

Saccharomyces cerevisiae ALRLFKDEPKWQAINRSNQADSLPVRKDYIALINQY-----
Arabidopsis thaliana  AMRLVGGPVWTPYNNRP--HDHLPARKAYMKKFLKVIQDRNIDASA
Mus musculus          AMRLVGGPVWTPYNNRP--ADHFDARVQYMSFLESTA-----
Homo sapiens          AMRLVGGPVWTPYNNRP--ADHFEARGKYVKFLAQTA-----
Klebsiella oxytoca    AIRIFDNPEGWIAQFTGDDIASAYPRLA-----
Bacillus anthracis    AIRIFVTTEGVPIY-----

*:*: *

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Figure 8. Sequence alignment of ARD homologs using ClustalOmega

The metal binding residues His88, His90, Glu94 and His133 are colored blue, the hydrophobic residues Phe84, Phe105, Phe135 and Ala145 found to be interacting with KMTB are colored red. Arg96 and Arg147 are colored green. Phe149 and Trp155 may be involved in conformational gating are shown in magenta. (All residue numbers refer to the MmARD sequence). The gap in the eukaryotic sequences (corresponding to the sequence APTAETVIAA in *Klebsiella*) is due to a shortened flexible loop D between strand C and helix E (Fig. 3).

Figure 9. Surface representation of MmARD showing residues likely involved in conformational gating

KMTB, Phe149 and Trp155 are shown in stick representation, Ni is shown as a green sphere and the water molecules shown as red spheres. Trp155 resides in the loop region preceding the long C-terminal helix, and may be involved in conformational changes upon substrate binding. Phe149 may be involved in the assisting Trp155 in conformational gating. a)

Overall structure, b) Overall structure showing the C-terminal helix and the loop preceding it in a cartoon representation, c) Active site structure showing Trp155, d) Active site structure showing Trp155 & Phe149.

Figure 10. Solution NMR data of Mn^{2+} , Fe^{2+} , Co^{2+} and Ni^{2+} -bound HsARD isozyms
Left: Upfield region of 800 MHz ^1H NMR spectra of Mn-HsARD (pink), Fe-HsARD (red), Co-HsARD (blue), and Ni-HsARD (green) showing methyl resonances of amino acid side chains ring current-shifted by nearby aromatic residues. The different spectral patterns indicate differences in side chain packing in hydrophobic cores. **Right:** Overlay of the 2D ^1H , ^{15}N HSQC spectra of Mn-HsARD (pink), Co-HsARD (blue), Fe-HsARD (red) and Ni-HsARD (green). Different shift patterns indicate differences in local hydrogen bonding and structure. All spectra were obtained at 25 °C, pH 7.0 at 800 MHz ^1H observe frequency. Reproduced with permission from Ref. 4. Copyright 2017 Oxford University Press.

Scheme 1.
Synthetic route to ARD substrates (Adapted from Ref. 46)

Scheme 2.

Model reactions showing feasibility of peroxy intermediates in Ni KoARD activity (Adapted from Ref. 46).

Scheme 3.

Proposed mechanism for inactivation of ARD via a radical intermediate generated by 1-electron transfer from substrate analog 5b (Adapted from Ref. 46).

Scheme 4. Proposed mechanisms of Ni-ARD and Fe-ARD using the chelate hypothesis

The results of incorporation of ^{18}O and ^{14}C labeling-studies are indicated by the red O atoms and the asterisk. Reproduced with permission from Ref 3. Copyright 2016 American Chemical Society.

Scheme 5. Proposed mechanism of Ni-ARD chemistry using small molecule model of Ni-KoARD active site
(Figure adapted from Ref. 94)

Scheme 6. Proposed mechanism of Fe-ARD chemistry using small molecule model of Fe-KoARD active site
(Figure adapted from Ref. 94)

Scheme 7. Proposed mechanism of Ni-KoARD and Fe-KoARD using computational studies
(Adapted from Ref. 19)

Scheme 8. Reactions catalyzed by oxalate decarboxylase (OxDC), oxalate oxidase (OxOx) and quercetinase (QueD)

Scheme 9. Proposed catalytic mechanism of oxalate oxidase

The protein ligands, His88, His90, His135, and Glu95 for Mn^{2+} are not shown. (Figure adapted from Ref. 67)

Scheme 10. Proposed catalytic mechanism of *Streptomyces* sp. FLA QueD

The protein ligands, His69, His71 and His115 for Ni^{2+} are not shown. (Figure adapted from Ref. 65)

Scheme 11. Proposed catalytic mechanism of homoprotocatechuate 2,3-dioxygenase which is an extradiol dioxygenase
(R = -CH₂COOH) (Figure adapted from Ref. 9)

Scheme 12. Ternary enzyme-substrate-dioxygen complexes for HPCD, HAD and HGDO observed by X-ray crystallography