Mechanistic Studies of Two Dioxygenases in the Methionine Salvage Pathway of *Klebsiella pneumoniae*[†,‡]

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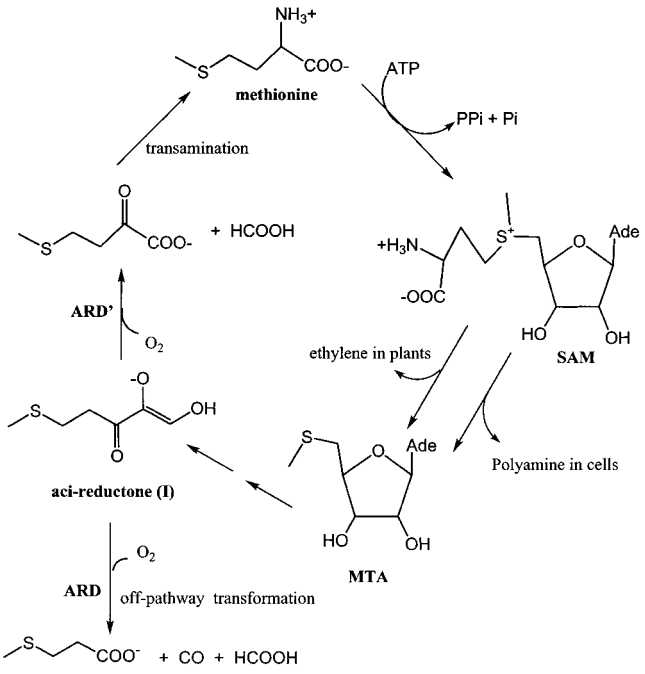
ABSTRACT: Two dioxygenases (ARD and ARD′) were cloned from *Klebsiella pneumoniae* that catalyze different oxidative decomposition reactions of an advanced aci-reductone intermediate, $CH_3SCH_2CH_2$-COCH(OH)═CH(OH) (**I**), in the methionine salvage pathway. The two enzymes are remarkable in that they have the same polypeptide sequence but bind different metal ions (Ni^{2+} and Fe^{2+}, respectively). ARD converts **I** to $CH_3SCH_2CH_2COOH$, CO, and HCOOH. ARD′ converts **I** to $CH_3SCH_2CH_2COCOOH$ and HCOOH. Kinetic analyses suggest that both ARD and ARD′ have ordered sequential mechanisms. A model substrate (**II**), a dethio analogue of **I**, binds to the enzyme first as evidenced by its λ_{max} red shift upon binding. The dianion formation from **II** causes the same λ_{max} red shift, suggesting that **II** bind to the enzyme as a dianion. The electron-rich **II** dianion likely reacts with O_2 to form a peroxide anion intermediate. Previous $^{18}O_2$ and ^{14}C tracer experiments established that ARD incorporates $^{18}O_2$ into C_1 and C_3 of **II** and C_2 is released as CO. ARD′ incorporates $^{18}O_2$ into C_1 and C_2 of **II**. The product distribution seems to necessitate the formation of a five-membered cyclic peroxide intermediate for ARD and a four-membered cyclic peroxide intermediate for ARD′. A model chemical reaction demonstrates the chemical and kinetic competency of the proposed five-membered cyclic peroxide intermediate. The breakdown of the four-membered and five-membered cyclic peroxide intermediates gives the ARD′ and ARD products, respectively. The nature of the metal ion appears to dictate the attack site of the peroxide anion and, consequently, the different cyclic peroxide intermediates and the different oxidative cleavages of **II**. A cyclopropyl substrate analogue inactivates both enzymes after multiple turnovers, providing evidence that a radical mechanism may be involved in the formation of the peroxide anion intermediate.

1,2-Dihydroxy-3-keto-5-(methylthio)pentene (**I**) is an advanced aci-reductone intermediate in the ubiquitous methionine salvage pathway that converts 5′-methylthioadenosine (MTA) to methionine. Two enzymes were previously identified in *Klebsiella pneumoniae* that catalyze oxidative decomposition reactions of **I** (Scheme 1) (*1*−*4*). In previous work, we described the discovery that both enzymes (called E2 and E2′ in previous papers) are obtained upon overexpression of a single *Klebsiella* open reading frame in *Escherichia coli*. The two enzymes (which we will now call ARD[1] and ARD′, for *a*ci-*r*eductone *d*ioxygenase) have the same polypeptide sequence and differ only in their metal content. ARD contains a single Ni^{2+} ion, while ARD′ contains a single Fe^{2+}. Both ARD and ARD′ react with substrate **I** and dioxygen but catalyze the formation of different products. ARD′ converts **I** to one molecule of the α-keto acid precursor of methionine and one molecule of

Scheme 1



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[‡] This paper is dedicated to the memory of Prof. Robert H. Abeles, who died on June 18, 2000. He is missed, and his students and colleagues will remember him with honor.

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[1] Abbreviations: ARD, aci-reductone dioxygenase; EPR, electron paramagnetic resonance; ES, enzyme−substrate complex; NMR, nuclear magnetic resonance; NADH/NAD^+, nicotinamide adenine dinucleotide (reduced/oxidized forms); KP_i, potassium phosphate buffer.

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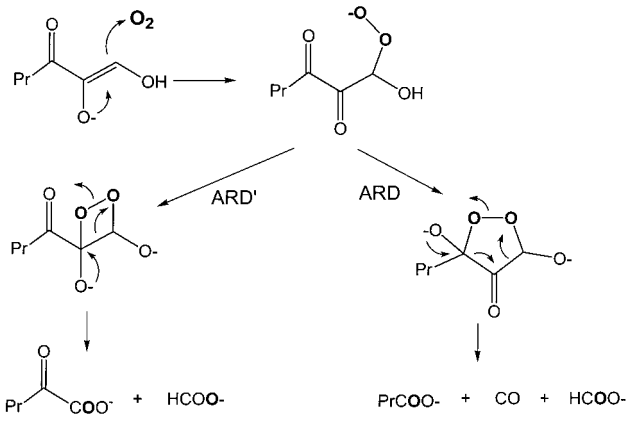
Scheme 2



formate and allows the recycling of methionine from MTA (*3*, *4*). ARD converts **I** to methylthiopropionate, CO, and formate and prevents the recycling of MTA to methionine (*1*−*4*). The two enzymes coelute from a size exclusion column but can be separated by ion-exchange and hydrophobic interaction chromatography, indicating some differences in their structures. They can be interconverted by metal removal and reconstitution with the appropriate metal ion. ^{18}O and ^{14}C tracer experiments have been used to establish that ARD and ARD′ are both dioxygenases. ARD incorporates $^{18}O_2$ into C_1 and C_3 of 1,2-dihydroxy-3-keto-1-hexene (**II**) and ARD′ incorporates $^{18}O_2$ into C_1 and C_2 of **II** (Scheme 2) (*2*, *3*). **II** is a model substrate for both ARD and ARD′.

The methionine salvage pathway recycles methionine from MTA. MTA is formed from *S*-adenosylmethionine (SAM) in the course of polyamine and ethylene biosyntheses (Scheme 1) (*5*−*6*). SAM is synthesized in turn from methionine and ATP. Polyamine and ethylene syntheses thus require continual input of ATP and availability of methionine, which is recycled through ARD′ activity. Polyamine is required for cell growth and proliferation (*5*). Ethylene is required for fruit and vegetable ripening (*7*). If the ARD activity predominates, it is expected that polyamine or ethylene biosyntheses cannot be maintained due to methionine depletion (*5*). The purpose of the off-pathway transformation of **I** catalyzed by ARD is unclear. The product of ARD activity, methylthiopropionate, is cytotoxic and has been implicated in the pathogenicity in plants (*8*). Interestingly, carbon monoxide, previously considered biologically relevant only as a toxic waste product, can increase the intracellular level of guanosine 3′,5′-cyclic monophosphate (cGMP) by activating guanylyl cyclase and is now considered a candidate for a new class of neural messenger (*9*, *10*). A dioxygenase has been identified in rat liver that exhibits the same activity as ARD′, converting **I** to 4-methylthio-2-ketobutyrate and formate (*2*). However, no eukaryotic dioxygenase has yet been found with activity corresponding to ARD, the oxidative release of CO from **I**.

The current work was undertaken in order to better understand how a single protein can give rise to more than one type of catalytic activity depending upon the metal present. Previously, we proposed a cyclic peroxy intermediate in the reaction catalyzed by ARD to explain observed regiochemistry in isotope labeling of the products generated (*2*, *3*) (Scheme 2). We have now performed further investigations of the catalytic mechanisms of ARD and ARD′ that provide insight into their substrate specificities, reaction intermediates, and kinetics.

MATERIALS AND METHODS

Enzyme Preparation. ARD and ARD′ were prepared according to published procedures (*1*). Enzyme concentrations were estimated according to the method of Bradford (*11*) using Coomassie Brilliant Blue G-250 (Bio-Rad) and bovine serum albumin as a standard.

Synthesis of Substrates. Chemicals used were of reagent grade or the best commercial grade unless otherwise specified. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer. ^1H shifts are reported relative to tetramethylsilane at 0 ppm for both ^1H and ^{13}C. The residual methyl signal of CD_3OD at 3.40 ppm was used as an internal ^1H chemical shift reference. Synthetic routes are outlined in Schemes 3 and 4. NMR chemical shifts as well as complete synthetic methodologies for substrates and substrate analogues described herein are to be found in the Supporting Information.

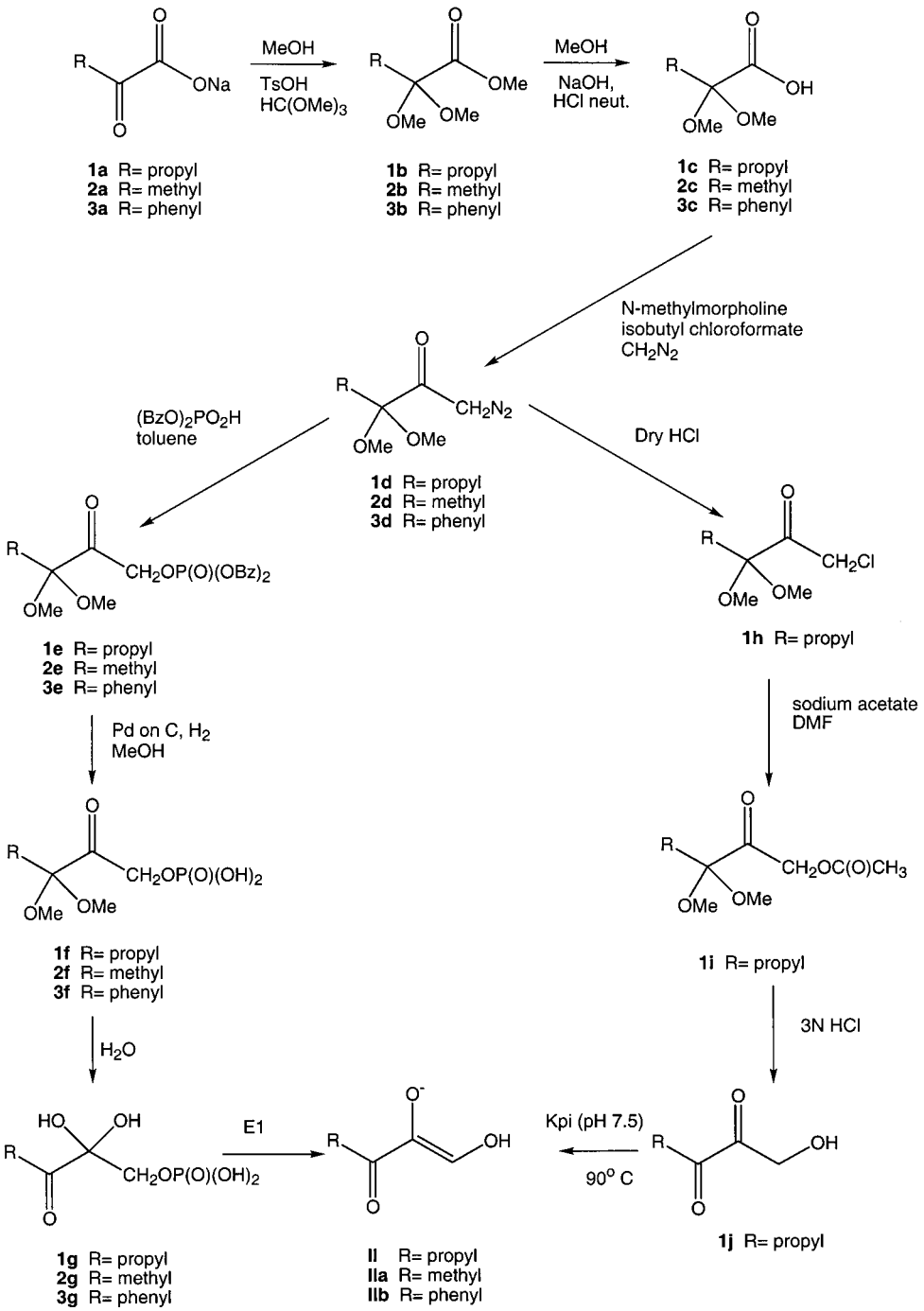
Analytical Methods. The absorption spectra of various aci-reductone substrates, their derivatives, and the change in spectra upon addition of enzyme were measured on a Perkin-Elmer UV−visible spectrophotometer under anaerobic conditions. Enzymatic oxidation of aci-reductone substrates and their derivatives was determined by measuring changes in absorption at characteristic absorption maxima (λ_{max}). Anaerobic conditions were obtained by repeated evacuation and flushing of substrate solution or enzyme solution with argon.

EPR spectra were recorded at liquid helium temperature on a Varian E6 EPR spectrometer in the laboratory of Dr. Graham Palmer, Rice University. The EPR conditions were collected using a modulation amplitude of 2 G, a time constant of 1 s, a power level of 1 mW, and temperature of 11 K. Radical concentration was determined by double integration of EPR signals, with reference to a Cu(II) EDTA standard. Electrospray mass spectra were obtained at the Mass Spectrometry Facility at Harvard University, Department of Chemistry. Metal contents of enzymes were determined by instrumental neutron activation analysis (INAA) at the Nuclear Reactor Laboratory, Massachusetts Institute of Technology. N-Terminal sequence analyses of enzymes were performed in Applied Biosystems 477A at Tufts University, Department of Physiology.

Formate Assays (*2*). Formate was converted to CO_2 by formate dehydrogenase in the presence of NAD. The concentration of formate was measured by monitoring the change of NADH absorbance at 340 nm.

O_2 Binding and Uptake. Oxygen binding to ARD and ARD′ was measured as follows. ARD or ARD′ [0.5 mL of 1.4 mM (7 μmol)] was placed in 50 mM KP_i, pH 7.5, buffer that was saturated with O_2. The solution was sealed in a 0.5 mL cuvette by a septum. The total amount of O_2 present was titrated with 50 mM aci-reductone (**II**) by injection. The concentrated enzyme solutions consumed 6.5 μmol of **II** and therefore was 1.3 mM in O_2. The O_2-saturated KP_i buffer alone was 1.4 mM O_2 as determined by oxygen electrode (Hansatech Instruments). It was concluded on the basis of this observation that the presence of either enzyme up to 1.4 mM does not significantly change the total amount of O_2 present in the buffer over that present in the saturated enzyme-free solution. It is unlikely, therefore, that the

Scheme 3



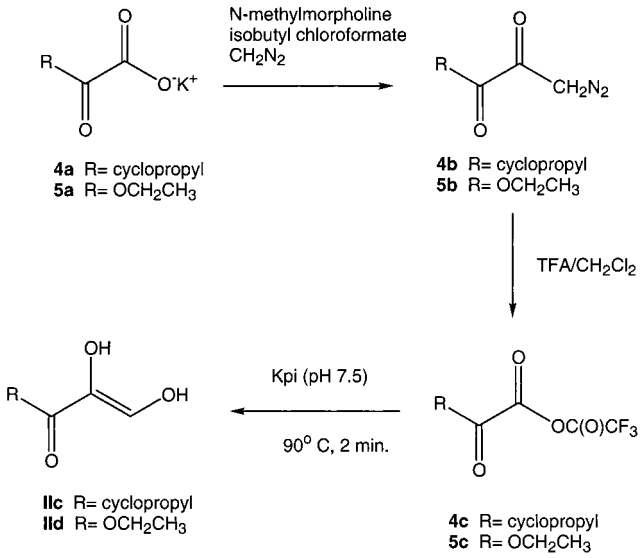
enzyme binds to O_2 in the absence of **II**. Dioxygen consumption and concentrations were monitored by means of an O_2 electrode at 25 °C. For O_2 consumption assays, the reaction mixture, in a total volume of 0.5 mL in the reaction chamber of the O_2 electrode, contained 50 mM KP_i, pH 7.5, 0.5 mM $MgSO_4$, 0.1 mM compound **II**, and the enzyme to be assayed.

α-*Keto Acid Assay* (*2*). The solution (0.48 mL) to be assayed was placed in a 1 mL cuvette along with 5 μL of 10 mM NADH. The solution was diluted to a total volume of 1 mL with distilled water, and the reaction was initiated by the addition of 50 units (5 μL) of bovine heart lactate dehydrogenase (Sigma). NADH uptake was monitored by absorbance at 340 nm. The reaction was allowed to go to completion.

Organic Acid Quantitation. Organic acid analyses were performed chromatographically. A Waters Aminex organic acid column (300 × 7.8 mm) was used and eluted with 5.0 mM H_2SO_4 at a flow rate of 0.5 mL/min. The column eluate was monitored by means of a refractive index detector (Waters Model R401). Quantitation of products was determined by determining peak areas.

CO Assay. Carbon monoxide formation was quantified by reaction with deoxyhemoglobin. An aliquot of the reaction mixture containing CO was added anaerobically to 0.1 mg of deoxyhemoglobin (prepared by the anaerobic reduction of met-aquohemoglobin with sodium dithionite) in 50 mM KP_i, pH 7.5. The absorption maximum of deoxyhemoglobin at 555 nm lost intensity, and new absorption maxima at 430 and 539 nm appeared. The Soret band shifted from 430 to

Scheme 4



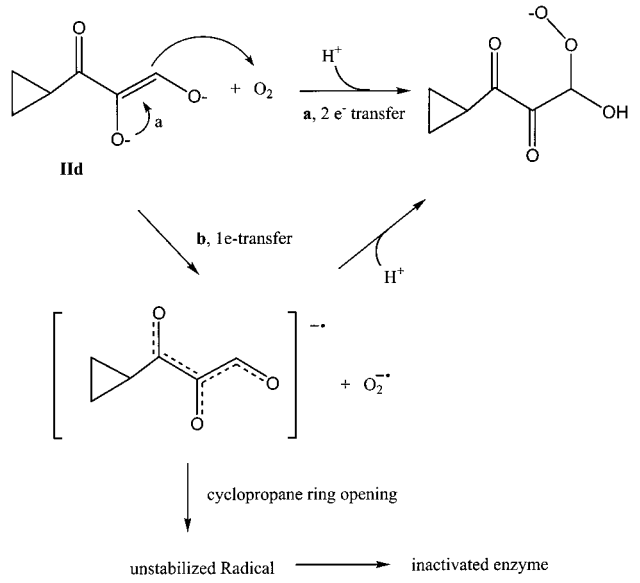
419 nm. The deoxyhemoglobin absorption spectrum was completely changed to the carbomonoxyhemoglobin absorption spectrum when the ratio of CO to deoxyhemoglobin reached 1:1.

Nonenzymatic Reaction of 2,3,4-Pentanetrione with H_2O_2. 2,3,4-Pentanetrione (eq 3, Scheme 6) was synthesized by a literature method (*15*). Pentanetrione (13.2 mg, 0.1 mol) reacts rapidly with 0.11 mmol of H_2O_2 in 100 mL of water to form 0.08 mmol of CO and 0.21 mmol of acetic acid. CO was quantified by binding to deoxyhemoglobin. The amount of acetic acid formed was determined by quantifying the sodium salt formed by addition of excess sodium bicarbonate after the reaction was complete. The removal of the solvent gave 2.1 mmol of pure sodium acetate as quantified by NMR. No other products were detected.

RESULTS

Purification and Characterization of ARD and ARD′. ARD and ARD′ were overexpressed and purified as described previously (*1*). The overproduced enzymes from *E. coli* have the same specific activities as those purified from *Klebsiella*. The molecular masses of overexpressed ARD and ARD′ estimated from SDS−PAGE gel electrophoresis (ca. 20 kDa) correspond to the masses deduced from gel filtration chromatography, indicating that both of the intact enzymes are monomers. The molecular masses of ARD, ARD′, and EDTA-treated ARD′ (apoenzyme) were determined by mass spectrometry to be 20 252 ± 20, 20 236 ± 20, and 20 192 ± 5 Da, respectively. The molecular mass of ARD calculated from the amino acid sequence is 20 184 kDa. The metal content of ARD and ARD′ was determined by instrumental neutron activation analysis. The results indicated that ARD and ARD′ contain approximately 1 atom of Ni and 1 atom of Fe, respectively (Table 1). No EPR signal was detected for either ARD or ARD′ under anaerobic or aerobic conditions, suggesting that both the Ni and Fe are divalent. ARD and ARD′ in the presence of aci-reductone substrate did not yield an EPR signal under either anaerobic or aerobic conditions, suggesting that metal-based redox changes during catalysis, if any, are transitory. The final homogeneous preparations of ARD and ARD′ are colorless and have no

Scheme 5



Scheme 6

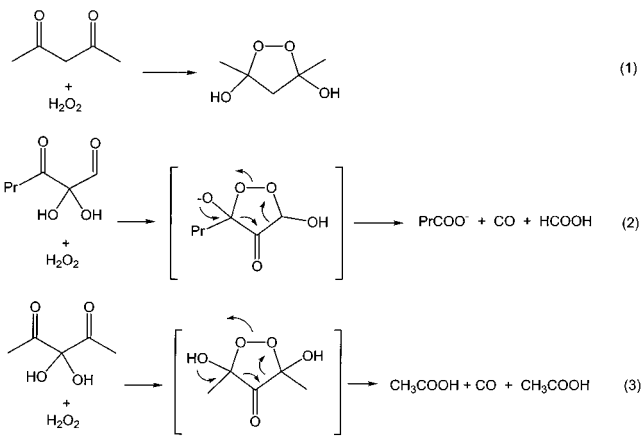


Table 1: Metal Contents of ARD, ARD′, Native ARD (Isolated from *K. pneumoniae*), and Apoenzyme by Instrumental Neutron Activation Analysis

	metal contents (mol of metal/mol of protein)		
proteins	Fe	Ni	Co
native ARD	0.1 ± 0.1	0.8 ± 0.2	0.3 ± 0.1
ARD	0.03 ± 0.02	1.1 ± 0.4	0.02 ± 0.01
ARD′	0.9 ± 0.2	0.06 ± 0.02	0.04 ± 0.01
apoenzyme	0.05 ± 0.02	0.2 ± 0.1	<0.001

absorbance at wavelengths of more than 300 nm, regardless of the absence and presence of 10 mM sodium borohydride (a reducing reagent).

The stoichiometries of the enzyme-catalyzed oxidations of **II** were examined. The results were summarized in Table 2. The data clearly demonstrate that ARD overexpressed in *E. coli* is indistinguishable from the native ARD in that both catalyze the oxidation of **II** to 1 equiv each of formate, CO, and butyrate, with concomitant consumption of 1 equiv of O_2. ARD′ catalyzes the oxidation of **II** to 1 equiv each of formate and 2-oxopentanate, with concomitant consumption of 1 equiv of O_2. Crude extracts of *K. pneumoniae* catalyze both reactions and generate mixed products. The products of the ARD′-catalyzed reaction of **II** with dioxygen are the same as those generated by the nonenzymatic reaction. As

Table 2: Stoichiometry of the Oxidations of **II**

reactants (nmol)			products (nmol)			
enzymes	**II**	O_2	2-oxo-pentanate	butyrate	CO	formate
nonenzymatic	80	81	70	<3	<5	85
cell extract	75	70	45	25	20	80
native ARD[a]	85	80	<5	75	80	91
ARD	84	75	<5	71	80	90
ARD′	84	73	63	<3	<5	89

[a]From *K. pneumoniae*.

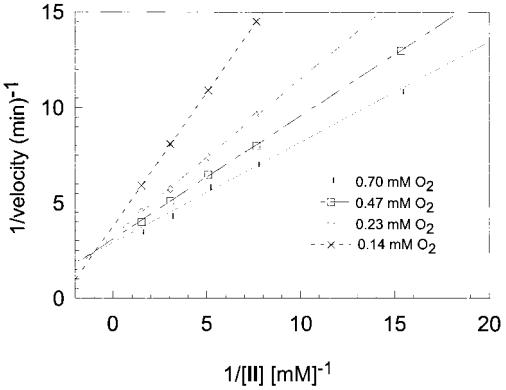


FIGURE 1: Double reciprocal plots of initial reaction velocity versus concentration of **II** in the presence of various constant levels of O_2 for ARD (Ni^{2+}). Experiments were performed in 50 mM KP_i buffer, pH 7.5. The reactions were initiated by the addition of 5 μL of a 0.1 mg/mL solution of ARD in a final volume of 0.5 mL. The enzymatic oxidation of **II** was followed by measuring the change in absorption at 305 nm.

Table 3: Kinetic Constants of ARD and ARD′

enzymes	ARD	ARD′
$K_m^{O_2}$ (μM)	110	47
K_m^{II} (μM)	50	52
K_s^{II} (nM)	25	53
k_{cat} (s^{-1})	5.0×10^2	2.6×10^2

discussed previously (*1*), we considered the possibility that ARD might make CO and butyrate by further decarbonylating the ARD′ product, 2-oxopentanate. However, ARD did not show any activity toward 2-oxopentanate ($k_{cat}/K_m < 10^{-4}$ M^{-1} s^{-1}), indicating that ARD and ARD′ do not catalyze sequential steps in the processing of substrate.

Kinetic Analyses. Kinetic studies were carried out by varying the concentration of one substrate in the presence of fixed concentrations of the second substrate. For ARD, double reciprocal plots, prepared by varying the concentration of **II** at fixed concentrations of oxygen, gave a series of straight lines with a single intercept along the 1/[**II**] axis (Figure 1). This is consistent with a sequential mechanism; i.e., both substrates must bind to the enzyme before any product is released. A secondary plot of intercepts and slopes from the first plot against O_2 concentration is linear and yields the pertinent kinetic constants (Table 3). Similar kinetics was observed for ARD′ (Figure 2). Both enzymes have comparable affinities for both O_2 and aci-reductone substrates and similar activities. Secondary plots are available in the Supporting Information.

Attempts to confirm the order of substrate binding to enzyme by product inhibition were unsuccessful. At concentrations of up to 1 mM, none of the reaction products

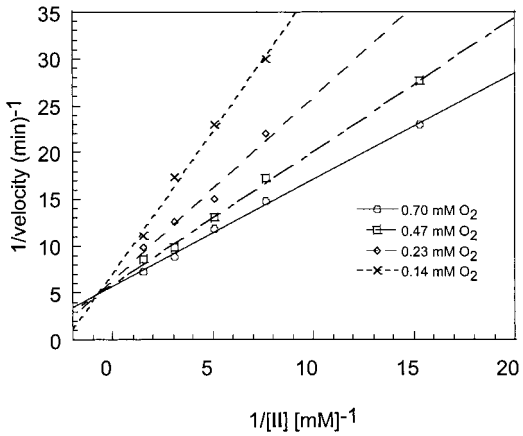


FIGURE 2: Double reciprocal plots of initial reaction velocity versus concentration of **II** in the presence of various constant levels of O_2 for ARD′ (Fe^{2+}). Experiments were performed in 50 mM KP_i buffer, pH 7.5. The reactions were initiated by the addition of 5 μL of a 0.1 mg/mL solution of ARD′ in a final volume of 0.5 mL. The enzymatic oxidation of **II** was followed by measuring the change in absorption at 305 nm.

(butyrate, 2-oxopentanoate, formate, or CO) inhibited either ARD or ARD′.

We examined the feasibility of the formation of binary E/**II** and E/O_2 complexes. O_2-saturated KP_i buffer was determined to contain 1.4 mM oxygen by oxygen electrode in the absence of ARD or ARD′. The O_2-saturated KP_i buffer in the presence of concentrated ARD or ARD′ (1.4 mM) was determined to also contain a total of 1.3 mM dioxygen (including free and enzyme-bound oxygen) by titration with **II**. As the presence of ARD or ARD′ did not increase the concentration of oxygen, there appears to be no enzyme-bound oxygen, or else O_2 binds to the enzyme with a K_d greater than 1 mM. Therefore, it is unlikely that oxygen binds to ARD or ARD′ to form the E/O_2 binary complex in the absence of **II**. On the other hand, **II** binds to ARD or ARD′ in the absence of oxygen as evidenced by the red λ_{max} shift of **II** upon the enzyme binding (vide infra). These studies are consistent with ARD and ARD′ exhibiting sequential kinetics and an obligatory order of substrate addition. The aci-reductone binds the enzyme first to give the E/**II** complex, which then binds oxygen to produce a ternary E/**II**/O_2 complex.

Substrate Specificity. The natural substrate for ARD and ARD′ is the 1,2-dihydroxy-3-keto-5-(methylthio)pentene anion (**I**) as shown in Scheme 1. **II**, a dethio analogue of **I**, was previously shown to be an alternative substrate (*3*). More substrate analogues have now been designed, synthesized, and tested in order to assess the relative importance of various functional groups on the substrate in binding and oxidative cleavage. The results are summarized in Table 4. Modified aci-reductones **IIa**, **IIb,** and **IIc** (see structures in Scheme 3) are substrates for both enzymes. The C1-phosphorylated version of **II** (**IIe**; see Table 4) and the conjugated ester **IId** (see structure in Scheme 4) do not bind nor are they substrates for either enzyme. These results suggest that the aci-reductone having the structure R−C(O)C(OH)═CH(OH) is essential for activity and binding. The phosphorylation of the hydroxy group may prevent **IIe** from binding to the enzyme's active site. The replacement of the C4 methylene by oxygen in **IId** could make it more difficult to form a dianion by raising the pK_{a2} of the terminal hydroxyl group,

Table 4: Characteristics of Aci-reductone Substrate Analogues[a]

substrate analogues	monoanion			dianion			relative activity (%)	
	pK_{a1}	λ_{max} (nm)	ϵ_{max} (M^{-1} cm^{-1})	pK_{a2}	λ_{max} (nm)	ϵ_{max} (M^{-1} cm^{-1})	ARD	ARD′
II	4.0	305	2.0×10^4	12.2	345	1.4×10^4	100	100
IIa	4.0	300	2.1×10^4	12.4	345	1.4×10^4	30	40
IIb	3.8	320	1.4×10^4	12.3	360	1.1×10^4	35	25
IIc	4.0	305	2.0×10^4	12.2	340	1.3×10^4	100	100
IId	4.2	290	1.8×10^4	13.0	320	8.0×10^3	<1	<1
IIe[b]	5.0	285	1.5×10^4				<1	<1

[a] Enzymatic oxidation of these compounds was determined by measuring the loss in UV adsorption. The wavelengths selected were those at which the compound gave the maximum absorption in the reaction mixture. The rate of degradation was determined by using known amounts of the enzyme and determining the number of nanomoles of substrate oxidized per minute. The rates were then expressed as a percentage of that obtained with **II** as the substrate. [b] Synthesis of compound **IIe**, 1-(phosphoryloxy)-2-hydroxy-3-ketohexane, is described in the Supporting Information.

if the substrate binds as a dianion, as suggested by spectroscopic observations (vide infra). Alternatively, this substitution might also interfere with the formation of a tetrahedral intermediate at the C_3 carbonyl. As shown in Table 4, the changes of the side chain or functional groups of **II** give parallel effects on substrate activity for the two enzymes, indicating that ARD and ARD′ have similar substrate specificities.

Spectroscopy of Enzyme−Substrate Complexes. The pK_{a1} and pK_{a2} values of **II** were determined to be 4.0 and 12.2, respectively, by titration with NaOH. The maximum absorbance of the aci-reductone substrates depends on its ionization state. As shown in Table 4, **II** exists as a monoanion at pH 7.4 and has a $\lambda_{max} = 305$ nm with a molar extinction coefficient (ϵ) of 20 000 M^{-1} cm^{-1}. The λ_{max} of **II** shifts from 305 to 345 nm with $\epsilon = 14\,000$ M^{-1} cm^{-1} when monoanionic **II** is further ionized to a dianion at pH 13.

The addition of equimolar amounts of ARD or ARD′ to **II** under anaerobic conditions rapidly causes a similar λ_{max} shift of **II** from 305 to 345 nm, as shown in Figure 3, upper panel (dashed line). This observation suggests that **II** may bind to the enzyme as a dianion. The chromophore generated by the ARD/**II** complex has a half-life of about 0.5 h under anaerobic conditions. The λ_{max} of the ARD/**II** complex disappears immediately upon the addition of O_2, producing approximately 1 equiv each of CO and HCOOH. The chromophore generated by complexation of ARD′ and **II** also disappeared immediately upon the addition of O_2, producing approximately 1 equiv each of α-keto acid and HCOOH. The addition of dioxygen to the premixed E/**II** complex gave the same products as the enzymatic reaction without premixing, suggesting that the chromophoric E/**II** complex is a real intermediate of the enzymatic reaction. To confirm that λ_{max} at 345 nm comes from **II** rather than the enzyme, phenyl-substituted **IIb** was used as a substrate. **IIb** has a λ_{max} at 320 nm ($\epsilon = 14\,000$ M^{-1} cm^{-1}) as monoanion and a λ_{max} at 360 nm ($\epsilon = 10\,000$ M^{-1} cm^{-1}) as a dianion (Table 4). λ_{max} shifts from 320 to 360 nm when an equimolar amount of ARD or ARD′ is added to **IIb** under anaerobic conditions, as shown in Figure 3, lower panel (dashed line). Again, dianion formation and enzyme binding cause the same λ_{max} shift of **IIb**. Furthermore, ES complexes differ in λ_{max} when the substrate is varied, indicating that the λ_{max} of ES is derived from the aci-reductone substrate. Attempts at measuring the rates of formation or oxidation of the enzyme−substrate complex were unsuccessful in that the reaction was complete within the dead time of our stopped-flow apparatus (∼5 ms).

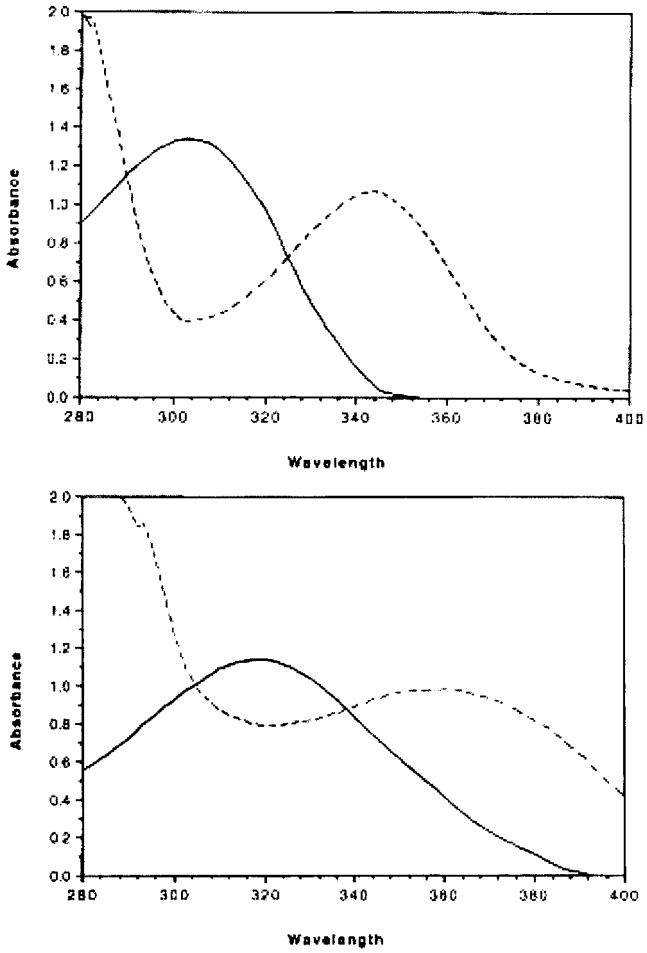
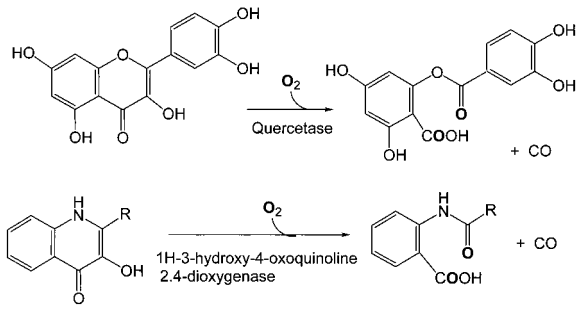


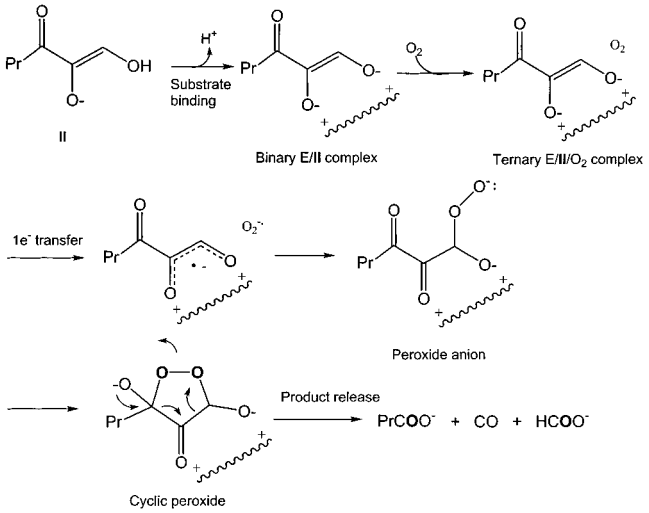
FIGURE 3: Absorbance maximum red shift of **II** and **IIb** upon enzyme binding. Upper panel: UV absorption spectra of **II** (solid line) and **II**−enzyme complex under anaerobic condition (dashed line). Both substrate and enzyme concentrations are 80 μM, pH 7.4, in KP_i. Lower panel: UV absorption spectra of **IIb** (solid line) and **IIb**−enzyme complex under anaerobic condition (dashed line). Both substrate and enzyme concentrations are 80 μM, pH 7.4, in KP_i.

Although we speculate that the active site metal in ARD and ARD′ may chelate with the dianion, this is by no means certain. We attempted to form the genuine chelate between Ni(II) or Fe(II) and the dianion of **II** in solution at pH 13. However, either no complex formed or else it was too unstable to characterize spectroscopically. The precise nature of the interactions stabilizing the formation of the substrate dianion in ARD will only be determined from high-resolution structural studies, now in progress.

Scheme 7



Scheme 8



Cyclopropyl Substrate Analogue **IIc** *as a Mechanistic Probe for a Radical Intermediate*. Cyclopropaneglyoxylic acid has been used as a probe for radical intermediates in lactate dehydrogenase (*13*). We wished to apply the same probe technique to ARD and ARD′ and so synthesized a cyclopropyl substrate analogue (**IIc**). If a radical is developed at the carbon α to the cyclopropyl ring, the cyclopropyl ring should open, forming a reactive radical that might inactivate the enzyme (Scheme 5). **IIc** exhibits the same λ_{max} as **II** (Table 4). A similar λ_{max} shift occurs when **IIc** is anaerobically titrated into the enzyme (data not shown). The chromophoric ARD/**IIc** or ARD′/**IIc** complexes are as stable as the ARD/**II** complex with a half-life of about 0.5 h. Incubation of **IIc** with ARD or ARD′ under anaerobic conditions does not result in inhibition. These results suggest that the ES complex is not a radical, consistent with the lack of an EPR signal for the anaerobic ES complex. However, **IIc** does irreversibly inactivate ARD after ca. 100 turnovers and ARD′ after ca. 20 turnovers in the presence of O_2, indicating that a radical intermediate might be formed or that a radical side reaction might take place after the formation of the ternary complex of enzyme, aci-reductone, and O_2.

Models for a Cyclic Peroxide Intermediate. The five-membered cyclic peroxide intermediate in Scheme 2 was proposed for the ARD-catalyzed oxidation reaction of **II** on the basis of ^{14}C and $^{18}O_2$ tracer experiments (*2*). We wished to examine the chemical competency of such a mechanism. It is known that H_2O_2 can attack diketones as a nucleophile to form cyclic peroxides. For example, hydrogen peroxide reacts with 2,4-pentanedione to form the stable 3,5-dimethyl-3,5-dihydroxy-1,2-peroxycyclopentane (eq 1, Scheme 6) (*14*). By analogy, hydrogen peroxide should react with the hydrated 1,2,3-hexanetrione to form a cyclic peroxide intermediate as in eq 2, Scheme 6. The synthesis of 1,2,3-hexanetrione was not successful in our hands, possibly due to the reactivity of the 1-carbonyl. However, 2,3,4-pentanetrione (eq 3, Scheme 6) was synthesized by a literature method (*15*). The pentanetrione reacts rapidly with 1 equiv of H_2O_2 in water to form 1 equiv of CO and 2 equiv of acetic acid (eq 3, Scheme 6), demonstrating that the cyclic peroxide decomposes into products analogous to those of the ARD-catalyzed reaction.

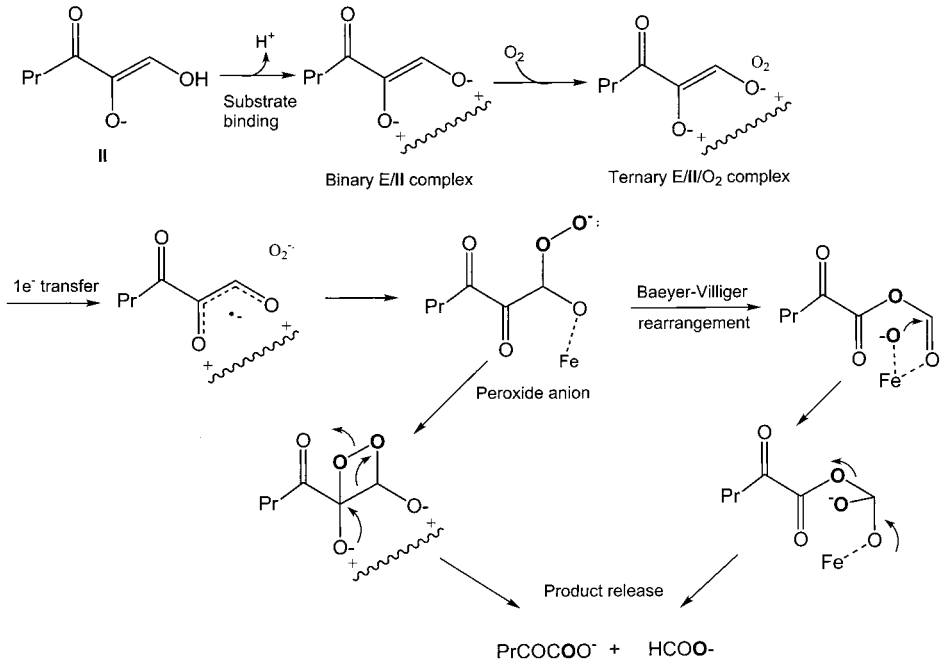
DISCUSSION

ARD and ARD′ are interesting (and perhaps unique) examples of two enzymes with the same polypeptide sequence that differ in multiple properties (e.g., enzyme activity, chromatographic separability) based solely on the difference in bound metal. The two enzymes are interconverted by removing and replacing the metals (*1*). Preliminary structural analysis using NMR methods suggests that ARD and ARD′ have similar, but not identical, structures (*16*; T. C. Pochapsky and H. Mo, unpublished results). While ARD′ catalyzes the on-pathway oxidation of I to the keto acid precursor of methionine, the ARD reaction provides a shunt out of the methionine salvage pathway, perhaps providing a mechanism for regulating methionine levels in vivo. It also provides a mechanism for CO production. CO has been implicated as a neurotransmitter molecule in mammals (*9*, *10*) and could conceivably serve a signal function in *Klebsiella* as well.

Nature of the Reactive Enzyme−*Substrate Complexes of ARD and ARD′*. The current studies demonstrate that ARD and ARD′ have comparable turnover numbers and substrate specificities. For both enzymes, the double reciprocal plots are linear and intersect at a single point, characteristic of a sequential mechanism in which all substrates must bind to the enzyme prior to product release (Figures 1 and 2). Linear intersecting initial velocities are seen in both random equilibrium and ordered sequential mechanisms. We were not able to distinguish these two mechanisms by product inhibition studies since none of the products (butyrate, 2-oxopentanate, formate, and CO) inhibit ARD or ARD′ at concentrations up to 1 mM. Many experiments have indicated rather conclusively that most dioxygenases bind O_2 only in the presence of substrate to form the ternary complex ESO_2 (*17*). Aci-reductone binds to ARD or ARD′ to form the ES complex in the absence of O_2 as evidenced in the red λ_{max} shift of bound aci-reductone. In contrast, oxygen was not observed to exhibit significant binding to either ARD or ARD′ in the absence of substrate. It is thus likely that the aci-reductone binds to the enzyme first, followed by O_2 binding to form the ternary complex.

The red λ_{max} shift that occurs upon anaerobic aci-reductone binding to ARD and ARD′ suggests that aci-reductone binds as a dianion to the enzymes. Substrate specificity studies suggest that dianion formation could be important for both binding and activity. **IId** has a higher pK_{a2} than **II** because of the replacement of α-methylene by oxygen (Table 4). The higher pK_{a2} makes it more difficult to form the dianion of **IId**, so it does not show either affinity for or activity with ARD or ARD′. The phosphorylation of the hydroxyl group

Scheme 9



at C_1 renders **IIe** without activity or affinity with ARD or ARD′. Aci-reductone dianions are electron rich and are susceptible to electrophilic attack by dioxygen, as confirmed by the nonenzymic reaction studies. The second-order rates of the nonenzymatic oxidations of monoanionic **II** at pH 7.5 and dianionic **II** at pH 13 were determined to be 0.12 M^{-1} s^{-1} and 8 M^{-1} s^{-1}, respectively. The nonenzymic oxidation of the dianion of **II** is about 66 times faster than that of the monoanion of **II** and yields the same products as the enzymatic reaction of **II** with O_2 catalyzed by ARD′.

Mechanistic Considerations: Radical vs Carbanionic Mechanism for ARD and ARD′ Activity. It is well-known that carbanions can react with oxygen to form peroxide anions. There are two common mechanisms for this reaction (Scheme 5). The peroxide anion can be formed by the direct electrophilic attack of triplet O_2 on a carbanion in a single step two-electron transfer process (*18*). The peroxide anion may also be generated via a radical mechanism in two steps involving one-electron transfers (*19*, *20*). We designed the cyclopropyl substrate analogue (**IIc**) as a mechanistic probe to distinguish between these two mechanisms. Anaerobic incubation of ARD or ARD′ with **IIc** does not inhibit either enzyme, indicating that no radical is formed from **IIc** in the absence of oxygen, an observation that is supported by the lack of an EPR signal for the ES complex. However, **IIc** slowly and irreversibly inactivates ARD after 100 turnovers and ARD′ after 20 turnovers in the presence of O_2, suggesting that a radical rearrangement can occur after O_2 is added, and the resulting relocated radical can inactivate the enzyme. This result suggests a radical mechanism for the peroxide anion formation. A one-electron transfer from the electron-rich aci-reductone dianion to oxygen would generate the aci-reductone radical anion and superoxide anion. These two radicals would then combine to form the peroxide anion. **IIc** does not inactivate the enzymes equivalently, possibly because the radical derived from **IIc** reacts with superoxide faster than it can rearrange and open the cyclopropane ring. If the initially formed radical is strongly resonance stabilized, this may also explain why the rearrangement is rather rare (*21*).

Evidence for a Cyclic Peroxide Intermediate in ARD and ARD′. In previous work, we used ^{14}C and $^{18}O_2$ tracer experiments (*2*) to determine the regiochemistry of incorporation of dioxygen into **II**. We found that ARD catalyzes the incorporation of $^{18}O_2$ into C_1 and C_3 of **II** but not into CO. C_2 is liberated as CO (Scheme 2). Two other dioxygenases (namely, eukaryotic quercetase and prokaryotic 1*H*-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase) catalyze similar oxidative cleavages (*22*−*26*). They incorporate both atoms of oxygen into C_2 and C_4 of their corresponding substrates, quercetin and 1*H*-3-hydroxy-4-oxoquinaldine, with C_3 being released as CO (Scheme 7). The structural similarity among the substrates suggests similar mechanisms for these three dioxygenases. Their product distribution by ^{14}C and $^{18}O_2$ tracer experiments seems to necessitate the formation of a five-membered cyclic peroxide intermediate. As discussed above, a five-membered cyclic peroxide intermediate is readily generated by nucleophilic addition of H_2O_2 to vicinal triketones, with decomposition to form 1 equiv of CO and 2 equiv of the expected acids.

Since most oxygenases require metal cofactors for activity, it has been argued that the coordination of oxygen to these metal cofactors may circumvent the spin restriction between the singlet ground-state organic molecules and the triplet ground-state oxygen, thereby resulting enzymatic activation of oxygen (*27*, *28*). This notion has largely been based on studies with monooxygenases. Studies with dioxygenases, however, indicate that metal cofactors may simply provide a means of binding oxygen to the enzyme or act as a general acid to activate substrate (*29*). Thus, dioxygen activation is less important to dioxygenases because their substrates are already relatively reactive toward triplet oxygen. For example, quercetin (*30*) and 1*H*-3-hydroxy-4-oxoquinaldine (*26*) undergo base-catalyzed oxidative cleavage and yield the same products as the respective enzyme-catalyzed reaction. No metal cofactor was identified for the 2,4-dioxygenase

associated with this chemistry (*26*). Aci-reductones undergo base-catalyzed oxidative cleavage in the absence of metal ions to give the same products as the ARD′-catalyzed reaction, indicating that the oxygen activation is not necessarily required for the ARD or ARD′ enzymatic reactions. It was recently discovered that another quercetin 2,4-dioxygenase that produces CO from quercetin and contains a Cu(II) cofactor is strongly inhibited by reductive ethylxanthation of the copper. This suggests that metal cofactors can play a critical role in some mechanisms for dioxygenase activity (*31*).

The above information enables us to propose a reaction mechanism for the oxidation of **II** by ARD (Scheme 8). Substrate **II** likely binds to the enzyme as a dianion (reflected in the red shift of λ_{max} of the E/**II** complex), possibly by ligating or chelating the divalent metal ion. Oxygen then binds to the E/**II** complex to form a ternary complex. One electron could then transfer from the dianion to O_2 to create the $\mathbf{II}^{\bullet-}$ and superoxide $O_2^{\bullet-}$ radical pair, which upon combination generates an organic peroxide anion. The Ni(II)-containing ARD then directs the attack of the peroxide anion at the C_3 carbonyl to form the cyclic five-membered peroxide intermediate. This intermediate then undergoes cleavage of two carbon−carbon bonds with the concomitant CO release.

ARD′ probably generates the peroxide anion in the same manner as ARD (Scheme 9). However, ARD′ directs the peroxide anion to attack the C_2 carbonyl to form the cyclic four-membered peroxide (dioxetane) intermediate, which then undergoes cleavage of two carbon−carbon bonds to form products. The formation of the dioxetane intermediate rationalizes the observation that both oxygen atoms are incorporated into C_1 and C_2 positions. Alternatively, the organic peroxide anion in ARD′ could undergo a Baeyer−Villiger-type rearrangement (Scheme 9). Migration of the acyl group, followed by decomposition, would give the observed products.

How the metal ions direct the different oxygen cleavage regiochemistries observed with ARD and ARD′ is unclear. Although the Ni^{2+} ion of ARD is paramagnetic, as demonstrated by the observance of strongly shifted resonances in the ^{1}H NMR spectrum of the resting enzyme (*16*), the absence of an EPR signal suggests a whole number electronic spin, consistent with octahedral coordination of the Ni^{2+}. The lack of evident color for the ARD enzyme (very low extinction coefficients for d−d transitions) is also typical of octahedral coordination of Ni^{2+} by hard ligands (*32*). If the Ni^{2+} is octahedrally ligated in the resting enzyme, it is coordinatively saturated, and chelation of the metal by the ligand might be unfavorable unless more than one ligand is solvent derived. However, the paramagnetically shifted ^{1}H ARD resonances are perturbed upon substrate binding, making it likely that there is some direct interaction between the substrate and metal ion (H. Mo, unpublished results).

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SUPPORTING INFORMATION AVAILABLE

Complete synthetic details as well as ^{1}H and ^{13}C NMR chemical shifts for substrates and intermediates and secondary plots of intercepts of slopes obtained from initial velocities of ARD and ARD′ as a function of O_2 concentration. This material is available free of charge via the Internet at http://pubs.acs.org.

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