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Structure and role for active site lid of lactate monooxygenase from *Mycobacterium smegmatis*

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Abstract: **Lactate monooxygenase (LMO) catalyzes the FMN-dependent "coupled" oxidation of lactate and O_2 to acetate, carbon dioxide, and water, involving pyruvate and hydrogen peroxide as enzyme-bound intermediates. Other α-hydroxy acid oxidase family members follow an "uncoupled pathway," wherein the α-keto acid product quickly dissociates before the reduced flavin reacts with oxygen. Here, we report the structures of *Mycobacterium smegmatis* wild-type LMO and a wild-type-like C203A variant at 2.1 Å and 1.7 Å resolution, respectively. The overall LMO fold and active site organization, including a bound sulfate mimicking substrate, resemble those of other α-hydroxy acid oxidases. Based on structural similarity, LMO is similarly distant from lactate oxidase, glycolate oxidase, mandelate dehydrogenase, and flavocytochrome b_2 and is the first representative enzyme of its type. Comparisons with other α-hydroxy acid oxidases reveal that LMO has a longer and more compact folded active site loop (Loop 4), which is known in related flavoenzymes to undergo order/disorder transitions to allow substrate/product binding and release. We propose that LMO's Loop 4 has an enhanced stability that is responsible for the slow product release requisite for the coupled pathway. We also note electrostatic features of the LMO active site that promote substrate binding. Whereas the physiological role of LMO remains unknown, we document what can currently be assessed of LMO's distribution in nature, including its unexpected occurrence, presumably through horizontal gene transfer, in halophilic archaea and in a limited group of fungi of the genus *Beauveria.***

Broad statement of impact: **This first crystal structure of the FMN-dependent α-hydroxy acid oxidase family member lactate monooxygenase (LMO) reveals it has a uniquely large active site lid that we hypothesize is stable enough to explain the slow dissociation of pyruvate that leads to its "coupled" oxidation of lactate and O_2 to produce acetate, carbon dioxide, and water. Also, the relatively widespread distribution of putative LMOs supports their importance and provides new motivation for their further study.**

Keywords: flavoenzyme; lactate metabolism; enzyme mechanism; protein crystallography; electrostatics; horizontal gene transfer

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Abbreviations and symbols: LMO, lactate monooxygenase; LOX, lactate oxidase; GOX, glycolate oxidase; MDH, mandelate dehydrogenase; FCB2, flavocytochrome b_2; HGT, horizontal gene transfer.

Additional Supporting Information may be found in the online version of this article.

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Dedication: Dedicated to Vincent Massey.

1. Introduction

An enzyme referred to as "lactate oxidase" was first discovered in *Mycobacterium phlei* in 1947,[1] and it was proposed that this yellow protein was a flavoenzyme that was able to convert lactate and molecular oxygen to acetate and carbon dioxide via a pyruvate intermediate. Subsequent characterizations[2] confirmed the enzyme to be FMN dependent[3,4] and to produce pyruvate as an enzyme-bound intermediate.[5] Although the name "lactate oxidase" persisted into the early 1990s, this acetate producing enzyme is now referred to as "lactate monooxygenase" (LMO), and "lactate oxidase" refers to a related flavoenzyme that converts lactate and molecular oxygen to pyruvate and hydrogen peroxide.[6]

LMO belongs to the α-hydroxy acid oxidase family of flavoenzymes including lactate oxidase (LOX), glycolate oxidase (GOX), flavocytochrome b_2 (FCB2), and mandelate dehydrogenase (MDH). The α-hydroxy acid oxidases have a common reductive half reaction in which the α-hydroxy acid is oxidized to an α-keto-acid and the enzyme-bound flavin is reduced. After much debate, the consensus is that this reaction involves a hydride transfer.[7–9] The oxidative half reaction (in which the flavin is reoxidized) can involve a reaction with molecular oxygen to produce hydrogen peroxide (e.g., for LMO, LOX, and GOX) or an electron transfer to a distant electron acceptor (e.g., for FCB2 and MDH). Among the family members reacting with oxygen, LMO is unique in that it proceeds along a "coupled pathway" [Fig. 1(A)], wherein rather than releasing the α-keto-acid as a product (in this case pyruvate), it remains in the active site, and after reaction with hydrogen peroxide undergoes an oxidative decarboxylation to produce acetate, carbon dioxide, and water.[6] In contrast, LOX and GOX proceed through an "uncoupled pathway" wherein the α-keto-acid product dissociates from the active site before the reduced flavin reacts with oxygen. For example, in LOX, lactate and oxygen are converted to pyruvate and hydrogen peroxide [Fig. 1(A)].

Working models for the active site of LMO have been based on the structures of LOX,[6,10] GOX,[11,12] FCB2,[13] and MDH,[14] all of which have a core TIM-barrel of 8 α-helices and 8 β-strands, bind FMN at the C-terminal end of the β-barrel, and include an often disordered loop (Loop 4) that covers the active site. Sequence and structural comparisons along with chemical modification[15–18] and site-directed mutagenesis studies[19–21] have identified seven conserved key active site residues in the family members [Fig. 1(B)], implying that their modes of ligand binding and catalytic mechanisms are very similar.

Given the high active site similarity between LMO and the other α-hydroxy acid oxidases, it was thought that its unique kinetics must somehow stem from subtle changes associated with differences in "second shell" residues near the LMO active site that could influence the flavin chemistry and substrate/product binding affinities. This made LMO an intriguing model for understanding and probing the fine-tuning of flavoenzymes, and Vince Massey (to whom this article is dedicated) and his research group carried out a series of studies in the 1990s to discover the origin of its unique kinetic properties.[6,19–23] Some of these site-directed mutagenesis studies were able to uncouple LMO, effectively converting its activity to that of LOX, but did so by greatly diminishing O_2 reactivity and/or changing conserved active site residues[21–23]—neither of which can reflect what differentiates LMO from the other α-hydroxy acid oxidases in nature. Indeed, LMO reacts readily with O_2, with the free enzyme having a rate constant of ~9000 s^{-1}, and the pyruvate complex being ~200-fold faster, at 180,000 s^{-1}.[5] While slowing the O_2 reactivity may uncouple the enzyme, this also has dramatic negative ramifications for catalysis and does not provide an understanding of the features differentiating LMO from LOX.

At that time, Vince Massey sought (in collaboration with our group) to gain insight through solving the LMO crystal structure, but while crystals were grown, no structure was solved.[24] Since then, with no structural information to guide further studies, our knowledge of LMO has been stagnant. Some insight has been provided by two recent structure-guided studies of LOX. Based on the authors' observation that "product off-rates appear to be dictated by partitioning of residues … from an active-site lid loop into bulk solvent,"[25] they mutated two "second shell" residues in the Loop 4 active site lid[26,27] and shifted its kinetic properties to be slightly more like LMO.

Here, we present the first direct structural information about any LMO, reporting both a 2.3 Å structure of *M. smegmatis* wild-type LMO (WT) and a 1.7 Å structure of a variant in which Cys203 is mutated to Ala (C203A). Chemical modification studies had implicated Cys203 as an active site residue,[28] but kinetic characterization showed this variant to "behave similarly to the wild-type enzyme in all properties examined,"[29] meaning it can be considered a reasonable surrogate for wild-type LMO. These structures reveal that LMO has the expected structural similarity to other family members with good positional conservation of the seven conserved active site residues. In addition to providing a solid structural foundation for further studies of LMO, we observe structural features that lead us to propose that LMO's unique kinetics are not a consequence of specific residue changes that influence the flavin chemistry, but are due to a difference in its dynamic Loop 4 which we suggest folds more stably than those of other family members and slows pyruvate release. The distribution of putative LMOs in nature provides intriguing evidence that LMO can at least in some cases bring a selective advantage to the organisms in which it is found.

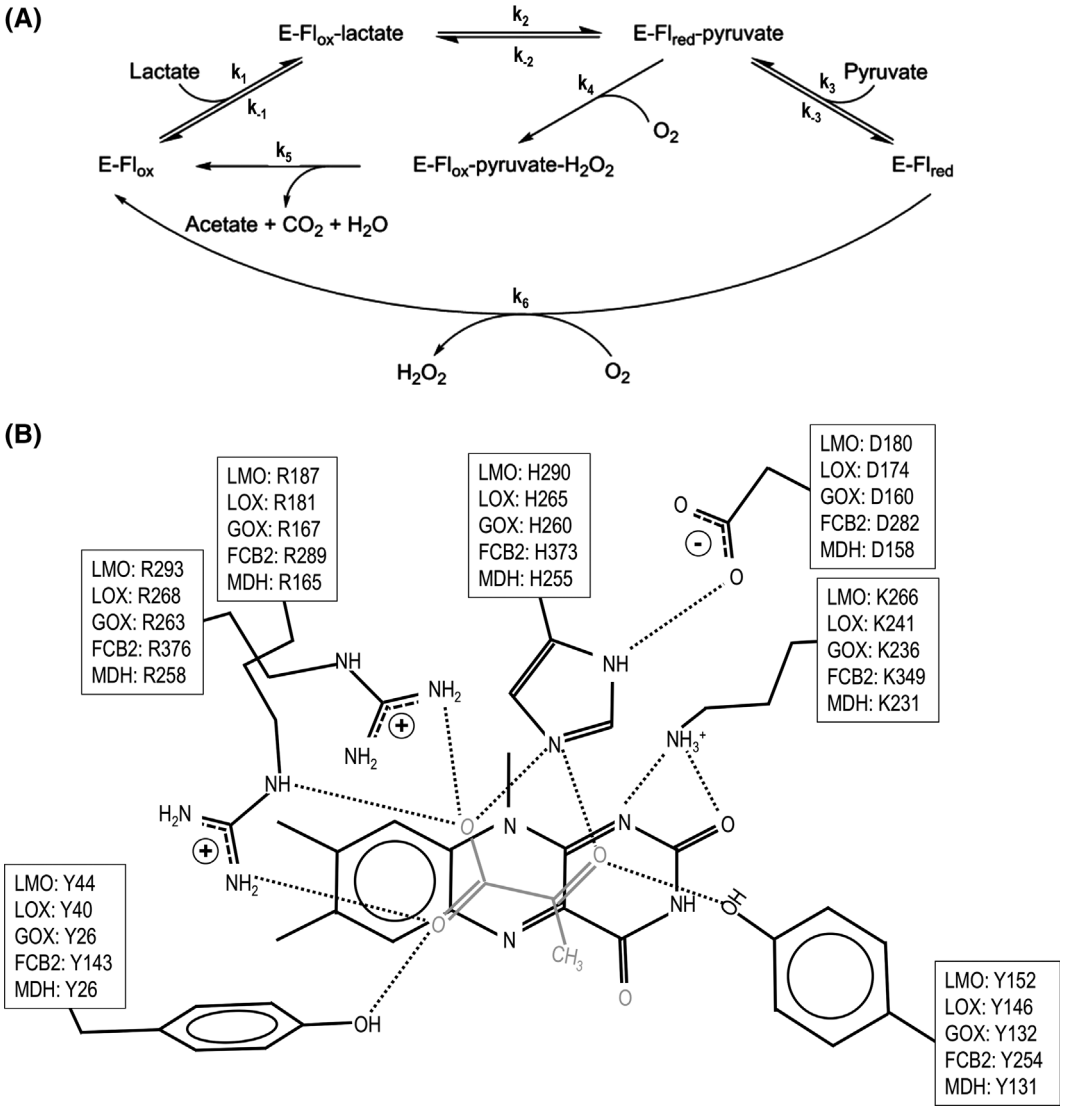

Figure 1. The LMO/LOX catalytic cycles and conserved active site of α-hydroxy acid oxidases. (A) The inner loop represents the "coupled pathway" observed for LMO where pyruvate is an intermediate and acetate and CO_2 are the final products. The outer loop represents the "uncoupled pathway" observed for LOX where pyruvate and H_2O_2 are produced by two uncoupled half-reactions. E, Fl_{ox}, and Fl_{red} represent the enzyme, oxidized flavin, and reduced flavin, respectively. Rate constants defined for each step are indicated. Adapted from Ref. 27. (B) Seven conserved residues in the active site of LMO, LOX, GOX, FCB2, and MDH are shown in front of the *si*-face of the flavin along with pyruvate (grey) as it is seen bound to LOX in PDB entry 2E77. Typical hydrogen bonds (dotted lines) are also shown. The residue numbers are based on *M. smegmatis* LMO, and PDB entries 2E77 (LOX), 2RDU (GOX), 1KBI (FCB2), and 1P4C (MDH).

2. Results and Discussion

2.1 Structure determination and crystal packing

Crystals of wild-type LMO were first grown and data, including the WT data set used here, were collected in the 1990s.[24] At that time and still today, the most similar structurally known protein had only ~35% sequence identity, and no success was achieved in determining the structure using either molecular replacement or SeMet MAD phasing (unpublished). The advance allowing us to solve the structure now was the development of MR-Rosetta[30] as a more powerful molecular replacement approach. MR-Rosetta was able to place four, well-ordered chains (A, B, C, and D), but this left significant weak but unmodeled density in a large swath of the crystal (Fig. 2). Two additional, weaker chains (E and F) were placed with Phaser (Materials and Methods), resulting in six total chains in the asymmetric unit. While the two additional chains have much weaker density, both are reliably placed based on clear, unbiased density consistent with the expected positions of their active site FMN and bound sulfate (Supporting Information, Fig. S1).

While we do not have a full understanding of this unusual crystal packing, we note that a fascinatingly similar phenomenon was reported for stefin B crystals.[31] In that example, the lattice of the crystal was

composed of five tetramer layers, four of which were well-ordered and one of which had two alternative positions that could both be modeled. Even though we have only been able to model a single position for chains E and F, as the electron density in that part of the crystal is so weak, it could be that there are other alternate positions for the chains that are not well-enough defined to model.

The refined model of LMO C203A at 1.70 Å resolution contains six chains in the asymmetric unit with R and R_{free} values of 16.9% and 19.4%, respectively (Table I). For five of the chains (A, B, C, D, and E), all 394 residues are sufficiently ordered to be modeled; for the sixth chain (F), the N-terminal methionine (residue 0 according to canonical LMO numbering[32]) is not well-ordered and only residues 1–393 are modeled. The refined model for WT at 2.30 Å resolution contains six chains in the asymmetric unit with R and R_{free} values of 21.8% and 27.2%, respectively (Table I). As in C203A, all 394 residues in chains A, B, C, and E are modeled and residues 1–393 are modeled in chain F. In WT, chain D is much less ordered and only residues 0–190, 203–216, and 261–393 are modeled.

Unless noted, all descriptions and analyses are based on the higher resolution structure of C203A. Given the stronger electron density of chains A–D, they are the only chains we consider reliable for defining details of the structures, so only they are described from here forward. The respectable R-factors of this structure imply that information for these well-ordered chains is not made less reliable by the presence of the less ordered chains. As chains A–D closely overlay with each other (Cα rmsd <0.4 Å), descriptions we provide based on chain A can be considered as true for all chains unless otherwise noted.

2.2 Structure of LMO

As expected for this family, the LMO monomer is organized into a β_8/α_8 TIM-barrel fold [Fig. 3(A)]. Each chain contains an FMN with sulfate ion bound above the *si* face of the isoalloxazine ring where substrate would bind [Fig. 4(A)]. LMO is reported to assemble into an octamer in solution.[32,33] In the crystals, each of the LMO chains in the asymmetric unit is part of a tetramer created by the crystallographic fourfold symmetry. Pairs of the unique tetramers further assemble into an octamer, with octamers formed from chains A and C, chains B and D, and chains E and F [Fig. 3(B)]. In WT, the modeled chain D tetramer is rotated ~22° with respect to the chain B tetramer, generating a surprising single instance of an alternate octamer interface that has looser packing (Supporting Information, Fig. S2). We cannot rule out that this alternate octamer has physiological relevance, but given that it has substantially looser packing and is only seen in a minority of the crystallized octamers, we posit that it is an artifact of the non-physiological crystallization conditions (at pH ~4.6 and high salt) that weakens the tetramer–tetramer interaction. As many of the related α-hydroxy oxidases function as tetramers,[34–36] it is to be expected that the tetramer is the stronger assembly, and the octamer would be more easily disrupted.

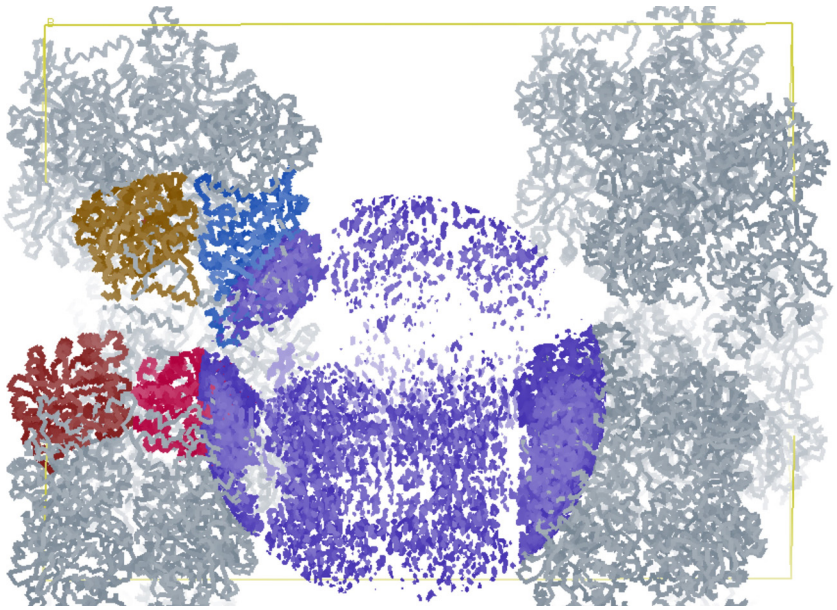

Figure 2. A swath of the LMO crystal has only very weak electron density. Shown are Cα-traces of the four well-ordered chains (mustard, blue, red, and burgundy) and their symmetry mates (grey) placed in the C203A unit cell (yellow box with the fourfold *c*-axis lying horizontally). Shown also is a sphere of the $2F_o$–F_c electron density map (blue; contoured at 1.3 ρ_{rms}) centered in the large swath of the crystal in which no model was included when calculating this map. The chain E and F models (at 80% occupancy) later placed in the weak density have average B-factors of ~110 Å^2 and ~120 Å^2, respectively, while chains A–D have average B-factors of ~30, ~45, ~45, and ~65 Å^2, respectively.

Table I. *Data Collection and Refinement Statistics for LMO Structures**

	WT	C203A
Data statistics		
Space group	$P42_12$	$P42_12$
Unit cell *a, b, c* axes (Å)	148.4, 148.4, 272.6	149.6, 149.6, 274.3
Resolution (Å)	77.49–2.30 (2.38–2.30)	30.90–1.70 (1.74–1.70)
Unique reflections	123105	33380 (24764)
Multiplicity	3.1	26.7 (27.5)
Average I/σ	10.4 (2.6)	15.5 (0.6)
R_{meas} (%)	10.2 (42)	18.2 (690)
Completeness (%)	91.0 (79.6)	99.9 (100)
$CC_{1/2}$	n.d.†	99.9 (22.7)
Refinement statistics		
Amino acid residues	2306	2363
Solvent atoms	1252	1464
Non-H atoms	19112	20104
RMS bonds (Å)	0.015	0.014
RMS angles (°)	1.6	1.4
ϕ, ψ favored (%) ‡	93.1	96.4
ϕ, ψ outliers (%) ‡	1.0	0.6
$<B_{protein}>$ (Å^2)	68	70
$<B_{solvent}>$ (Å^2)	45	52
R_{work} (%)	21.8	16.9
R_{free} (%)	27.2	19.4
PDB code	6DVI	6DVH

* Numbers in parentheses represent data for the high-resolution shell.
† Not determined. Data were collected and processed 20 years ago, and images are not available for reprocessing. The statistics are taken from Ref. [24].
‡ Ramachandran statistics defined by Molprobity.[80]

In the active site of LMO, very clear electron density defines the positions of all seven residues conserved among the α-hydroxy acid oxidases: Tyr44, Tyr152, Asp180, Arg187, Lys266, His290, and Arg293 [Fig. 4(A)]. Tyr44, Arg293, Arg187, and His290 hydrogen bond with the ordered sulfate. Asp180 hydrogen bonds with His290-Nδ1 and is well-positioned to stabilize the presumed active site base, His290, when it becomes protonated after abstracting a proton from the substrate.[2,6] Lys266 hydrogen bonds with FMN N1- and O2-atoms and can stabilize the anionic semiquinone.[2,6,19,37] Two additional ordered waters form hydrogen bonds with the sulfate and Tyr152.

2.3 Relationships to other structurally known proteins

A structural similarity search performed using the DALI server[38] shows that LMO is roughly equally similar to other members of the α-hydroxy acid oxidase family including GOX (rmsd ~1.4–1.9 Å, 33%–36% sequence identity), MDH (rmsd 2.0 Å, 32% sequence identity), LOX (rmsd ~1.6–1.8 Å, 31%–33% sequence identity), and FCB2 (rmsd 1.7 Å, 31% sequence identity). Since LMO is as distant from each of the other α-hydroxy acid oxidases as they are from each other, we conclude it not evolutionarily closer to LOX. Furthermore, this indicates that LMO truly is a distinct branch of the family and that even with all the structures that are known and have been solved from structural genomics projects, the structure we report here is still the first structure representing the LMO clade.

For carrying out a structure-based sequence alignment (Fig. 5), using the DALI results, we selected a representative structure for each enzyme type with the greatest amount of its chain ordered. These were *Aerococcus viridans* LOX (2E77),[10] human GOX (2RDU),[12] *Saccharomyces cerevisiae* FCB2 (1KBI),[13] and *Pseudomonas putida* MDH (1P4C).[14] The resulting alignment shows good conservation of structural elements and active site residues across these enzymes. As we discuss further below, a notable feature is that there is substantial variation in both the length and structure of what has been called Loop 4, a segment between strand β_4 and helix α_4 that has been found to be disordered in many structures.

An overlay of LMO with sulfate bound onto LOX and GOX structures having pyruvate and oxalate, respectively, bound in the active sites shows that the FMN and side chain positions are quite similar [Fig. 4(B)]. Only one side chain, that of Tyr44 in LMO, has notable variation, being shifted ~2.5 Å compared to LOX Tyr40 to hydrogen bond with the bound sulfate. In addition to the seven key, conserved active site residues, LOX has an additional tyrosine (Tyr215) that contributes to the active site [Fig. 4(B)] and forms a hydrogen bond with the pyruvate product. This tyrosine is part of the variable Loop 4, and neither LMO nor GOX have a functionally or structurally equivalent residue.

Each of these nearly identical active sites is well set up to carry out the same chemistry: the

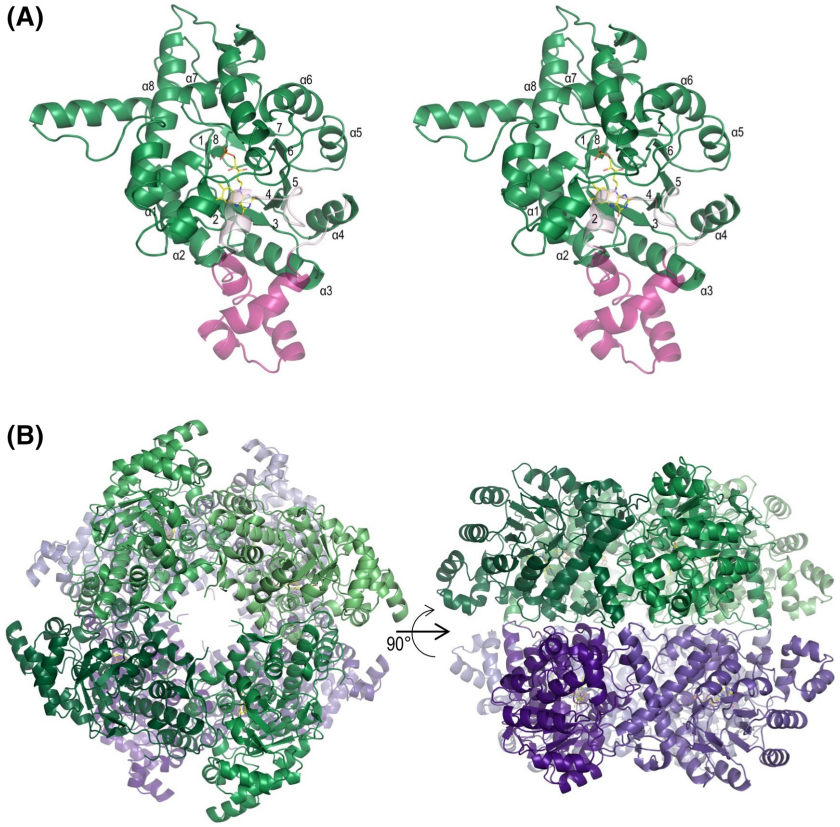

Figure 3. Tertiary and quaternary structures of LMO. (A) Stereoview of one subunit of LMO (C203A chain A) showing FMN (yellow carbons) and viewing into the barrel. The TIM-barrel α-helices (α1–α8) and β-strands (1–8) are labeled. The segment connecting β4 and α4 is semitransparent with the αD conserved portion in light pink and the Loop 4 variable portion in dark pink. (B) Shown are views of the C203A LMO octamer looking down the crystallographic fourfold axis (left) and perpendicular to it (right). The two tetramers forming the octamer are based on chain A (shades of green) and chain C (shades of purple). See Supporting Information, Figure S2 for an alternate octamer observed for chains B and D in the wild-type LMO crystals.

FMN-dependent oxidation of an α-hydroxy acid substrate. In all structures, the putative catalytic base His (His290 in LMO, His265 in LOX, and His260 in GOX) is well-oriented to abstract a proton from substrate, and an Asp (Asp180 in LMO, Asp174 in LOX, and Asp160 in GOX) is nearby to stabilize the protonated His. Likewise, a Lys (Lys266 in LMO, Lys241 in LOX, and Lys236 in GOX) is positioned to stabilize negative charge at the N1/O2 locus of the flavin during catalysis. The sulfate we see in the LMO active site appears to be a reasonable substrate mimic, as the hydrogen bonds with sulfate and ordered waters observed in LMO match well with interactions with ligand observed in LOX and GOX. Specifically, the carboxylate oxygens of the ligands bound in LOX and GOX overlay well with two of the oxygens of sulfate in LMO and form equivalent hydrogen bonds to those sulfate makes with Arg187 and Arg293 in LMO. Similarly, hydrogen bonds between O2 of pyruvate with residues equivalent to His290 and Tyr152 mimic the hydrogen bonds made with a sulfate oxygen and ordered water, respectively, in LMO. Given its high similarity with related structures, we do not see this structure as a source of further insight into the reaction chemistry, and so we do not comment further on that topic.

2.4 *Major differences occur in Loop 4 that covers the active site*

It has been noted that the α-hydroxy acid oxidases have substantial sequence and structural divergence in a segment between β4 and α4, referred to as Loop 4 (Fig. 5).[12,39] In all structurally characterized family members including LMO, Loop 4 functions as a lid or flap, covering the active site pocket when folded (i.e., closed) and exposing the active site opening when unfolded (i.e., open) (Fig. 6 and Supporting Information, Fig. S3). Thus, movement of this loop is necessary for substrate binding and product release.[10,12,25–27,40] For LMO, LOX, and GOX, the opening of the loop not only makes the active site accessible, but also exposes a highly positive surface patch surrounding the active site opening that can promote catalysis by actively drawing the negatively charged α-hydroxy acid substrate (e.g., lactate) into the active site [Fig. 6(A) and Supporting Information, Fig. S3]. Such an electrostatic guidance of substrate binding has been described for GOX,[41] and one of the

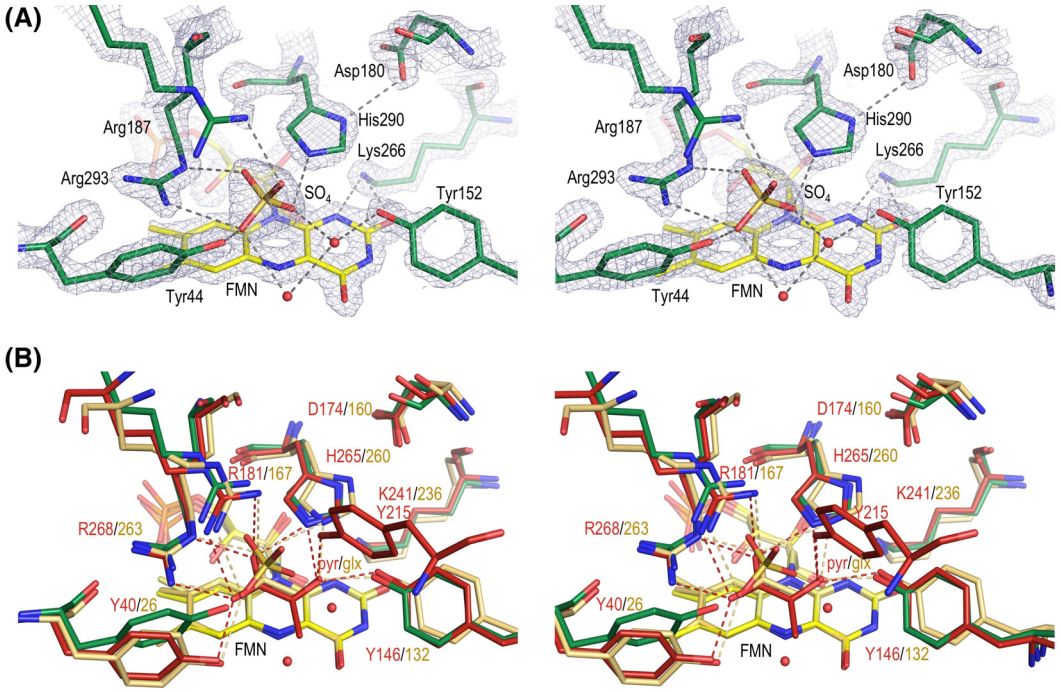

Figure 4. Active site of LMO. (A) Electron density map quality and active site of LMO. Stereoview of the LMO active site residues (green carbons), FMN (yellow carbons), sulfate, and ordered waters (red spheres) with $2F_o–F_c$ electron density (grey; contoured at $3.5\rho_{rms}$). Select hydrogen bonds (dashed line) are shown. Arg187 has an alternate conformation, but only the A conformation (50%) interacting with the sulfate is shown. The view roughly matches that shown in Figure 1(B) schematic. (B) Active site overlay of LMO with LOX and GOX. Stereoview of the LMO active site (colored as in Fig. 4) compared with LOX with bound pyruvate (red carbons; PDB entry 2E77) and GOX with bound glyoxylate (tan carbons; PDB entry 2RDU). Hydrogen bonds (dashed line) between active site side chains and the ligand in LOX (red) and GOX (beige) are shown.

best-studied examples of this phenomenon is copper superoxide dismutase.[42] Interestingly, when Loop 4 is closed and blocks substrate binding, it also alters the electrostatic potential surface in this part of the protein, so substrate will not be attracted [Fig. 6 (B) and Supporting Information, Fig. S3].

For the other α-hydroxy acid oxidases, partial or complete disordering of Loop 4 is seen in structures with no active site ligand and also in many structures containing a bound product or substrate/product analog.[10,11,40] This implies that Loop 4 readily unfolds, and that it undergoes folding and unfolding transitions associated with substrate binding and product release. With the structure of LMO in hand, a notable feature is that, even though only a sulfate is bound at the active site, the Loop 4 of LMO is fully ordered and structured similarly in all chains, including the two less ordered chains (E and F). Also, compared to LOX and GOX, the LMO Loop 4 is both longer (Fig. 5) and folds quite differently [Fig. 7 (A)]. In *M. smegmatis* LMO, Loop 4 is 49 residues long and adopts a compact association of four short α-helices to create a small hydrophobic core [Fig. 7 (B)]. A DALI search[38] of the folded Loop 4 segment alone revealed no proteins that contain closely similar folds.

Given that Loop 4 must unfold for product release, we asked if the Loop 4 subdomain of LMO might be more stably folded than those of LOX and GOX such that Loop 4 dynamics could dictate the speed of pyruvate release and LMO's kinetics. First, we asked if differences in loop lengths in the representative structures were characteristic, and found that LMOs do consistently have longer Loop 4s than LOXs and GOXs (Table II). Then, we sought to assess if the longer LMO Loop 4 might also be more stably folded. There is a large body of literature indicating that buried surface area is correlated to stability in proteins,[43–46] so we calculated for each representative enzyme how much surface area is buried when going from an unfolded Loop 4 to the fully folded Loop 4 active site lid (Table II). We found that folding of the LMO Loop 4 buries ~1500 and ~900 Å^2 more surface than those of LOX and GOX, respectively (4750 vs. 3290 Å^2 and 3870 Å^2). This difference is due to the surface buried internal to Loop 4, consistent with the loop being more stably folded as a co-dependent subdomain. Forming a central hydrophobic core of Loop 4 in LMO are Leu204, Phe213, Phe217, Leu228, Ala235, Phe238, and Trp239 [Fig. 7(B)]. Many of these core residues, along with residues forming the interface between Loop 4 and the rest of the protein, are relatively well conserved across representative LMOs [Fig. 7(C)], implying that the Loop 4 folding pattern and interaction with the rest of LMO are also well-conserved.

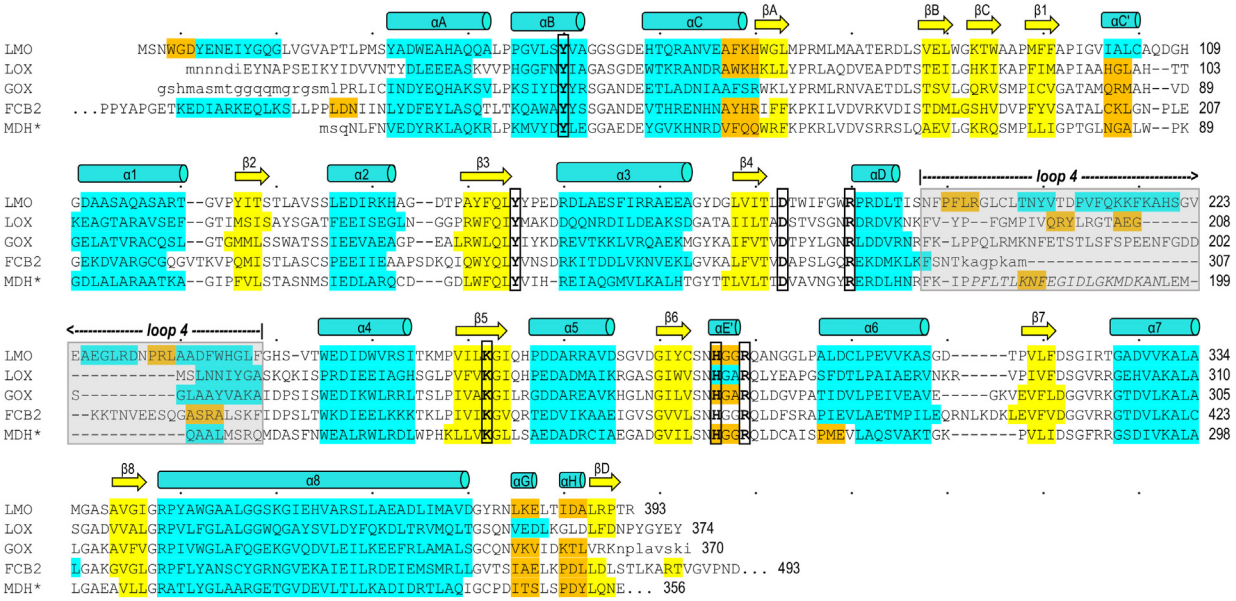

Figure 5. Structure-based sequence alignment of LMO with representative α-hydroxy acid oxidase family members. LMO (WT sequence, structure from C203A chain A) is shown first followed by LOX (PDB entry 2E77 chain B), GOX (2RDU chain A), FCB2 (1KBI chain A), and MDH* (MDH-GOX chimera with chimeric region from spinach GOX italicized; 1P4C chain A). Conserved active site residues (bold, black outline) and residues in α-helices (cyan), 3_{10}-helices (orange), and β-strands (yellow) are indicated. Secondary structural elements conserved among all family members are named in a manner consistent with naming conventions for other family members.[6,11,39] Loop 4 (gray shading) is an active site lid that has been seen to undergo order/disorder transitions in other α-hydroxy acid oxidases; when ordered, it covers the active site channel but is not structurally conserved between family members. Dots above the LMO sequence indicate every tenth character and a number is given for the residue at the end of each line. Residues in lower case letters are disordered in the respective structure.

Taking these observations together, we hypothesize that the LMO Loop 4 has a greater propensity to be closed, especially when substrate or product is bound, and is the key factor that slows pyruvate release and leads LMO to follow the coupled reaction pathway.

2.5 Other studies involving Loop 4 residues support this hypothesis

An older pair of observations supporting this hypothesis is that treatment with fluoro-dinitrobenzene (FDNB) blocked LMO activity through the modification of Cys104 and Cys203 and that these residues

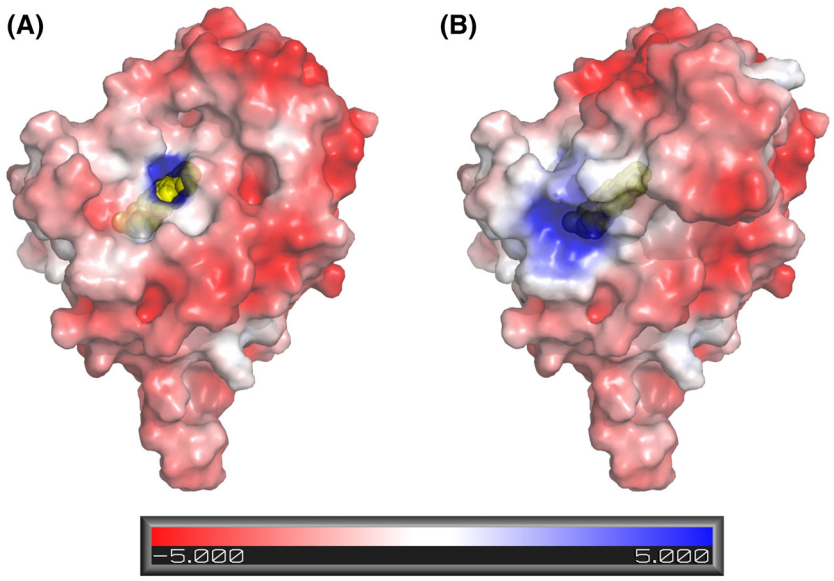

Figure 6. The open LMO active site electrostatically attracts substrate and Loop 4 folding blocks active site access. (A) Molecular surface and electrostatic potential of LMO with Loop 4 removed. FMN in the active site (yellow spheres) is visible through an opening. The surface electrostatic potential (semitransparent) is scaled from red to blue as shown. (B) Same as panel A, but with Loop 4 present.

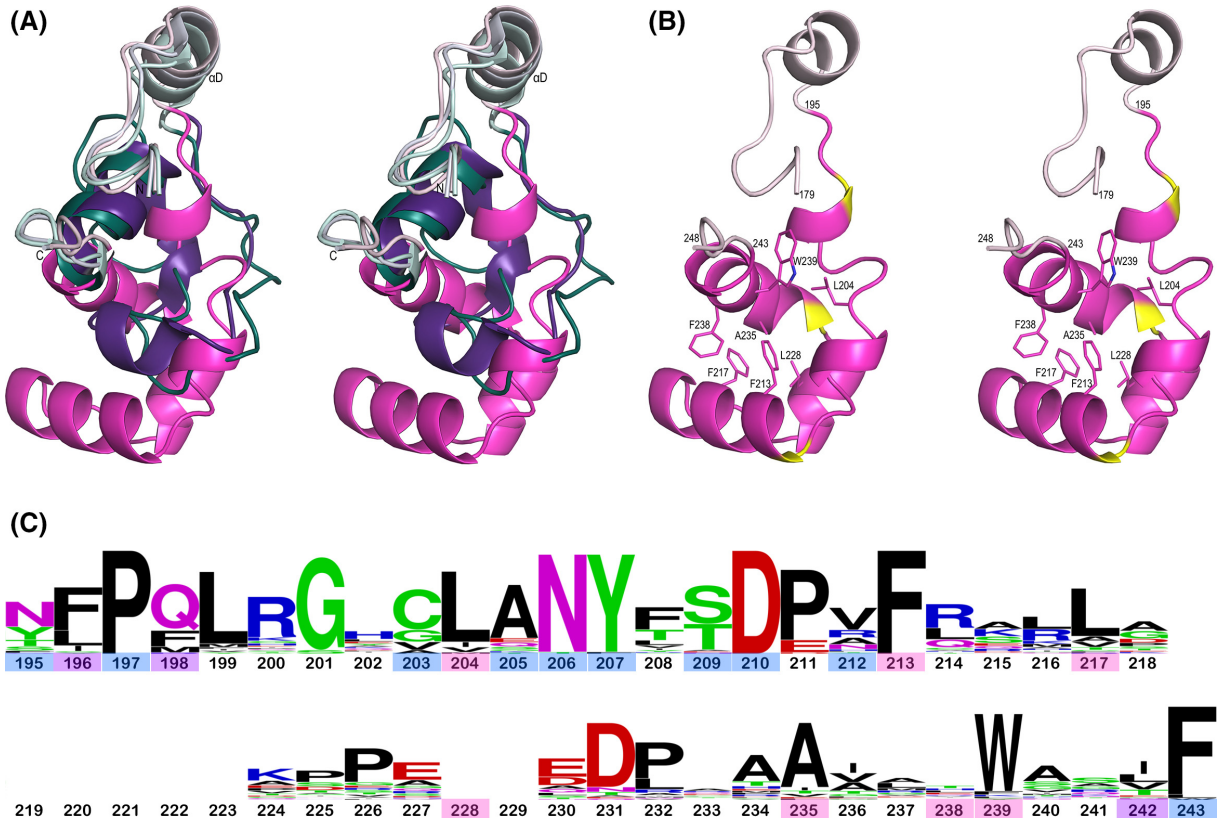

Figure 7. Comparison of Loop 4 in LMO, LOX, and GOX. (A) Stereoview of the chain between β4 and α4 of the LMO monomer (pink tones) overlaid with the LOX–pyruvate complex (purple tones; PDB entry 2E77) and the GOX–glyoxylate complex (teal tones; 2RDU). The N- and C-termini are marked and segments between β4 and α4 are highlighted with the more spatially conserved parts at the beginning and end, including αD, in light colors and the varying Loop 4 portion in dark colors. The LOX and GOX Loop 4 segments follow similar paths to each other. (B) Stereoview of the LMO chain between β4 and α4 (orientation and ribbon coloring as in A, but with three helix-capping Pro residues in yellow) with seven central core side chains shown as sticks and labeled. (C) WebLogo plot based on Loop 4 sequences from 48 representative LMOs using *M. smegmatis* LMO numbering. Residue numbers are colored based on the side chain role in packing: central core of Loop 4 (pink), interface between Loop 4 and the rest of LMO (blue), or both core and interface (purple). At 49 residues long, *M. smegmatis* LMO has the longest Loop 4 with the most common length being 42 residues; the 7 residues not present in most LMOs are blank spots in the WebLogo. The conservation of Gly201 which adopts a glycine-preferred ϕ,ψ-conformation and Pro197, Asp210/Pro211, and Asn231/Pro232 which form helix N-capping motifs imply a strong conservation of these structural elements across all putative LMOs. The very conserved Asn206 and Tyr207 pack at the interface with the rest of LMO, with the Asn206 side chain hydrogen bonding to backbone atoms 135-N and 129-O, and Tyr207 packing between Phe163 and Loop 4 core residues.

could be protected by inhibitor binding.[28,32] With the structure of LMO in hand, we now see that neither cysteine directly contributes to substrate binding or catalysis. Cys104 lines the flavin binding pocket where it is mostly buried behind the C7-methyl of the flavin. Cys203 is in Loop 4 and is buried at the interface between Loop 4 and the rest of the protein. Importantly, both cysteines are positioned where Loop 4 folding and closure should protect them from modification, and also where a large modification (such as FDNB

Table II. *Surface Areas (SA) Related to the Folding of Loop 4**

	LMO	LOX	GOX
PDB entry analyzed	6DVH	2E77	2RDU
SA unfolded Loop 4 (Å^2)	6240	3630	4710
SA buried internal to Loop 4 (Å^2)	2570	1140	1660
SA Loop 4 interface with protein (Å^2)	1160	1190	1190
SA protein interface with Loop 4 (Å^2)	1020	960	1020
Total SA buried upon Loop 4 folding (Å^2) †	4750	3290	3870
Range of Loop 4 lengths (residues)	39–49 (49)	27–29 (29)	17–38 (38)

* See Materials and Methods for details of calculations.
† Sum of the three previous entries.
‡ Number in parenthesis is the loop length in this representative structure.

produces) would hinder enzyme activity by blocking proper folding of Loop 4. That inhibitor binding protected the residues from modification supports our hypothesis that Loop 4 is strongly closed in the presence of an active site ligand and could hinder product release. Also, the facile modification of the two Cys residues in the absence of an active site ligand implies the loop is dynamic enough in the absence of ligand to allow for rapid binding of substrate.

In newer work, as mentioned in the Introduction, there are two recent site-directed mutagenesis studies that probed interactions involving Loop 4 residues in LOX. In both cases, the results provide experimental support for our hypothesis, because they directly link Loop 4 thermodynamics with the pyruvate dissociation rate. In LOX, Tyr215 from Loop 4 contributes to the active site (Figure 4B) and forms an additional hydrogen bond with the pyruvate product. In a Tyr215Phe mutant (Y215F), pyruvate release was decreased by approximately sevenfold.[26] Similarly, mutating LOX Tyr191, another conserved Loop 4 residue, to Phe, Leu, or Ala slowed the pyruvate release step by ~5-, 19-, and 19-fold, respectively.[27] In both cases, it was concluded that the mutations shifted the Loop 4 dynamics to favor a closed conformation and that this hindered pyruvate release.[26,27] While these mutations slowed pyruvate release [k_3 in Fig. 1(A)], it did not become rate limiting, and no mutants had measurable flux through a coupled pathway. This is not surprising, because none of them came close to matching the ~3000-fold slower k_{off} observed in LMO.[5]

2.6 LMOs exist beyond bacteria

As far as we could find in the literature, LMO has exclusively been studied from mycobacteria since it was first identified in 1947.[1,2,19,33] However, the physiological role of LMO remains unknown. With an extensive number of genomes now having been sequenced, a database search can provide a more complete picture of the range of organisms that appear to have LMO (Fig. 8). Selecting all proteins with ~50% or higher sequence identity with *M. smegmatis* LMO yields a phylogenetic tree that groups all these putative LMOs more closely with each other than with LOX, GOX, FCB2, and MDH, confirming that with this cutoff the putative LMOs are at least much more similar to each other than to any of the other known α-hydroxy acid oxidase enzymes.

Using this criterion, putative LMOs are most abundantly represented in Actinomycetales, an order of Gram-positive and generally aerobic bacteria including *Mycobacterium*, *Nocardia*, *Streptomyces*, *Rhodococcus,* and *Frankia.*[47] LMO are also identified in bacteria from α- and γ-proteobacteria, plantomycetes, pseudocardiales, thermophilia, geodermatophilales, and bacilli. Surprisingly, putative LMO sequences much like those in bacteria were found in a single fungal genus, *Beauveria,* and one archaeon, *Halopiger salifodinae.* As noted below, further investigation supports the conclusion that these sequences are reliably present in these nonbacterial sources and not due to contaminants, making it natural to suggest that they were derived through horizontal gene transfer (HGT).

In the case of *Beauveria,* a putative LMO gene that has a prokaryotic gene structure (i.e., lacks introns) is found in all sequenced strains of *B. bassiana* and its sister species, *B. brongniartii* (also known as *Cordyceps brongniartii*) (Supporting Information, Fig. S4), but is not found in other closely related entomopathogens such as Cordyceps (e.g., *C. militaris* and *C. confragosa*) and Metarhizium (e.g., *Metarhizium album* and *Metarhizium robertsii*). These *Beauveria* proteins group closely with those from the human pathogens, *Mycobacterium abscessus*, responsible for a multidrug-resistant diseases in humans,[48] and *Tsukamurella pulmonis*, commonly associated with infections in immunocompromised humans[49] (Fig. 8 and Supporting Information, Fig. S4). While it may seem odd that these human pathogenic bacteria and insect pathogenic fungi would have closely related LMOs, *T. pulmonis* and many other mycobacteria have been isolated from insects,[49,50] and a variety of insects, including bedbugs and bloodsucking insects, have been implicated in the spread of mycobacteria.[50] Thus, HGT between ancestors of these bacteria and of *B. bassiana* could have occurred in an insect carrier. In a potentially similar HGT scenario, the related entomopathogenic fungus *Metarhizium anisopliae* acquired a phosphoketolase from bacteria that is necessary for insect virulence and aids survival of the fungus in the insect haemocoel.[51,52] This raises the possibility that that LMO was acquired because it confers a selective advantage to *Beauveria* fungi.

H. salifodinae is a halophilic archaeon and its putative LMO groups closely with that of the halophilic proteobacterium *Salincola salarius* (Fig. 8). A BLAST search using the *H. salifodinae* protein as query retrieves an extensive set of putative LMOs from a range of halophilic archaea (Supporting Information, Fig. S5). It has been observed that anaerobic and thermophilic bacteria share an unusually large number of genes with archaea and that HGT is especially rampant among archaea and bacteria found in similar environments,[53,54] making it plausible that the presence of LMO in these organisms presents another example of this evolutionary phenomenon.

2.7 Outlook

This long overdue structure determination of an LMO provides a piece that has been missing in studies of the α-hydroxy acid oxidase family. It lays a solid foundation for reinvigorating structure–function studies on this enzyme, as well as providing a specific, testable hypothesis that a more stable folding of Loop 4 is responsible for LMO's unique coupled reaction that directly produces acetate and carbon dioxide and does

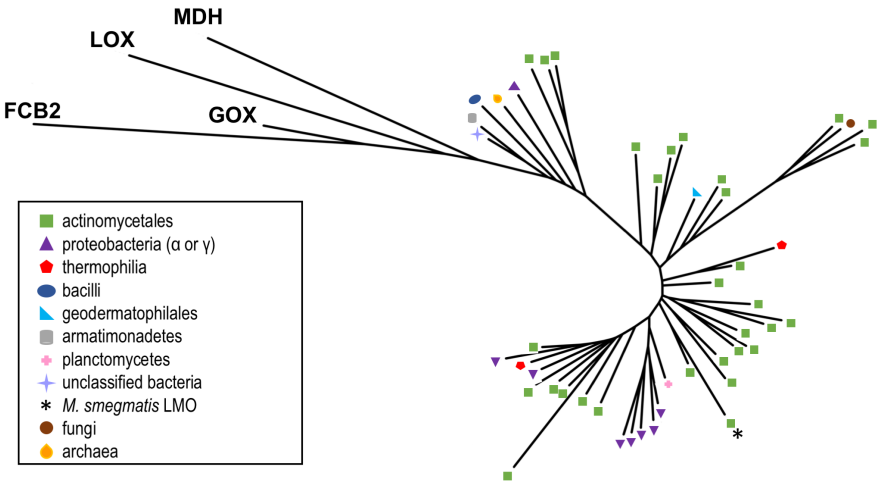

Figure 8. Relatedness tree and distribution of putative LMOs and representative α-hydroxy acid oxidases. An unrooted tree is shown for the 48 representative putative LMO sequences along with proteins from representative PDB entries from LOX (PDB entry 2E77), GOX (2RDU), MDH (1P4C), and FCB2 (1KBI). The branches for the putative LMO sequences are annotated by symbols according to the organism where they are found, with a key in the figure indicating which symbols correspond to which organisms.

not release pyruvate as a product. Further, our analysis of the distribution of putative LMOs in nature, including the varieties of bacteria in which it is found and its surprising presence in a fungus and in halophilic archaea, provides new motivation to discover the physiological role it plays in these organisms.

3. Materials and Methods

3.1 Crystallography and structure determination

Recombinant LMO (WT and C203A) from *M. smegmatis* was provided by Dr Vince Massey (University of Michigan). The protein sample was shipped at 4°C as precipitated protein in 1.0 M acetate, pH 5.4. Before crystallization, the protein was transferred to 5 mM HEPES pH 7.0, 10 μM PMSF and concentrated to 20 mg/mL. Aliquots were frozen and stored at −80°C.

The proteins were crystallized at 4°C in hanging drops with a reservoir solution of 0.1 M sodium citrate pH 4.6, 0.4 M lithium sulfate, and 0.4 M ammonium sulfate. For WT, crystals were transferred into a series of artificial mother liquors containing 0%, 5%, 15%, and 25% glycerol and plunged into liquid nitrogen. Data were collected in 1996 at CHESS station A1 using $\Delta\varphi = 0.5°$ rotation and processed using DENZO.[55]

For C203A, crystals were transferred to an artificial mother liquor containing 25% glycerol and plunged into liquid nitrogen. Data were collected at beamline 5.0.2 at the Advanced Light Source using $\lambda = 1.0$ Å, $\Delta\varphi = 0.25°$ rotation, 0.25 s exposure, collecting 360° total. Data were processed and handled using XDS[56] and the CCP4 program suite.[57] A $CC_{1/2}$ cutoff criteria[58] of ~0.2 after merging was used to define the resolution cutoff of 1.70 Å, and a random 5% of reflections in resolution bins were marked for cross-validation.

Largely due to the unusual observed crystal packing, these structures proved challenging for molecular replacement and were tackled in an atypical, multistep manner. The phase problem was initially solved by molecular replacement using MR-Rosetta with default settings[30] using the 2.3 Å WT dataset and a homology search model generated by HHpred.[59] The initial solution contained four chains with 1687 residues built and R/R_{free} of 0.34/0.38. All manual modeling building and refinement was done in Coot[60,61] and Phenix,[62] respectively.

To have plausible crystal packing, it was clear that further chains had to be placed even though there was only very weak density in a swath of the crystal along the *c*-axis (Fig. 2). Attempts to place these chains using the WT data were not successful, but we were able to place the chains using the higher resolution data from the C203A crystal and a combination of MR-Rosetta, Autobuild, and Phaser. After obtaining a higher resolution C203A dataset, the existing WT model was used as a placed model for model-building using MR-Rosetta with the 1.7 Å C203A dataset. The resulting solution from MR-Rosetta contained four chains with 1522 residues built and R/R_{free} of 0.26/0.27. This solution was used to seed Autobuild,[63] which led to no discernable changes in the models (and we presume was not a necessary step). After some additional refinement, the four chains were fed into Phaser[64] as an already placed model, and molecular replacement using C203A Chain A—without its FMN and bound sulfate or waters—as a search model and with the packing criteria turned off gave a solution that contained two additional chains. These chains were in the weaker

electron density region of the crystal, and a confirmation of their correctness was provided by a difference map that had strong positive peaks in both chains corresponding to where FMN and sulfate should be found (Supporting Information, Fig. S1).

This model containing the four well-ordered chains (A, B, C, and D) and two less ordered chains (E and F) was further refined. Because the electron density is so weak for chains E and F, we suspect there may be alternate ways for these chains to pack that support crystal formation. For this reason, we allowed the occupancies of these chains to refine, and set the occupancy for both chains at 80% based on the average occupancy of their atoms. As modeled, chains E and F each closely overlay with the well-ordered chains, with both having a Cα rmsd of <0.4 Å compared to chain A. Water molecules were manually placed based on having electron density ≥ 4.0 ρ_{rms} in F_o–F_c maps and ≥ 0.9 ρ_{rms} in $2F_o$–F_c maps and reasonable hydrogen bonding interactions. Further refinement of the C203A structure, with riding hydrogens and TLS using one group per chain (i.e., six total TLS groups), led to the final model (statistics in Table I).

The LMO WT structure was resolved by molecular replacement using a nearly final C203A structure as the search model, following the same methods as above for modeling and refinement. As chain D was clearly different in the WT structure (Supporting Information, Fig. S2), before refinement progressed, that chain was replaced by the model for chain D that was obtained from the initial WT MR-Rosetta solution, which was correctly positioned.

3.2 Phylogenetic studies

We carried out a BLAST search in March 2018 using the WT LMO sequence as the query and generated 1000 putative LMO sequences. The sequences all had *e*-values above ~8×10^{-112} and sequence identity of ~50% or higher. To generate a smaller set of representative sequences, the 1000 sequences were clustered into 48 groups using CD-Hit[65] with sequence identity cutoff of 70%. The representative 48 sequences output by CD-Hit and the four sequences of representative PDB structures of GOX, LOX, MDH, and FCB2 were aligned. To assess the reliability of putative LMO sequences in *Beauveria* and archaea, we carried out additional BLAST searches using the *B. bassiana* LMO sequence and *H. salifodinae* LMO sequence as query, respectively. In the *B. bassiana* search, all fungal sequences included had ~95% sequence identity or higher and the most similar bacterial sequence had ~70% sequence identity. This set was aligned with *Beauveria* sequences provided by Dr Claudio Valero (University of Wageningen), WT LMO, and LMO sequences from *Mycobacterium abscessus* and *Tsukamurella pulmonis*. From the *H. salifodinae* search, we generated a set of archaeal sequences that included all archaeal sequences that were more similar to *H. salifodinae* LMO than the most similar bacterial sequence; this included sequences of ~60% sequence identity and higher. They were aligned with WT LMO. In all cases, sequences were aligned with MUSCLE,[66] and trees were generated using PhyML[67] and Interactive Tree of Life.[68]

3.3 Electrostatic potential and surface area calculations

Electrostatic potentials were calculated using the PDB2PQR server[69] with the AMBER force field and Adaptive Poisson–Boltzmann Solver (APBS) server.[70,71] Parameters for FMN were generated using PRODRG[72] with atomic partial charges from Rohr et al.[73] Electrostatic potentials were rendered using Pymol[74] in conjunction with the APBS plugin.[71]

The solvent-accessible surface areas of folded proteins, both with and without Loop 4, were calculated using Areaimol.[75,76] The solvent-accessible surface areas of the unfolded Loop 4 were calculated using the upper and lower bound model proposed by Creamer *et al.*,[77–79] with the values reported in Table II being the average between the calculated upper and lower limits. For these calculations, the residues defining the beginning and end of Loop 4 were set, respectively, as the equivalent to LMO residues 195 and 243 based on the residues for which representative structures for GOX, LOX, FCB2, and MDH deviated in a structural overlay. The range of Loop 4 lengths in the LMO family was determined based on the same 48 sequences used for the phylogenetic tree. For the LOX and GOX families, the ranges were determined using COBALT alignments generated from a BLAST search using the representative enzyme (2E77 for LOX; 2RDU for GOX) and the top 100 (down to ~60% sequence identity) or 250 sequences (down to ~70% sequence identity), respectively. In the case of GOX, two sequences were removed, one of which was annotated as "low-quality protein" and one of which was an incomplete sequence.

The WebLogo[80] plot for LMO was generated using the same 48 sequences used for the phylogenetic tree. Residues in the core and/or interface of Loop 4 were determined based on both visual assessment and by comparing the residue-level solvent exposed surface reported by DSSP[81] in the absence and presence of the rest of the protein.

3.4 Accession numbers

Coordinates and structure factors for LMO WT and C203A have been deposited in the Protein Data Bank with the accession numbers 6DVI and 6DVH, respectively.

Acknowledgments

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the wild-type structure. They also thank Dale Tronrud for his assistance in tackling this challenging molecular replacement, and Claudio Valero and Jan van Kan (University of Wageningen) for their guidance, insights, and unpublished sequences related to the LMO genes found in *Beauveria*, and also Michael Freitag and Joey Spatafora (Oregon State University) for helpful conversations.

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