OXALOACETATE HYDROLASE: THE C-C BOND LYASE OF OXALATE SECRETING FUNGI.

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Oxalate secretion by fungi is known to be associated with fungal pathogenesis. In addition, oxalate toxicity is a concern for the commercial application of fungi in the food and drug industries. Although oxalate is generated through several different biochemical pathways, oxaloacetate acetylhydrolase (OAH) catalyzed hydrolytic cleavage of oxaloacetate appears to be an especially important route. Below, we report the cloning of the Botrytis cinerea oah1 gene and the demonstration that the disruption of this gene results in the loss of oxalate formation. In addition, through complementation we have shown that the intact B. cinerea oah1 gene restores oxalate production in an Aspergillus niger mutant strain, lacking a functional oah1 gene. These observations clearly indicate that oxalate production in A. niger and B. cinerea is solely dependent on the hydrolytic cleavage of oxaloacetate catalyzed by OAH. In addition, the B. cinerea oah1 gene was overexpressed in E. coli and the purified OAH was used to define catalytic efficiency, substrate specificity and metal ion activation. These results are reported along with the discovery of the mechanism-based, tight-binding OAH inhibitor 3,3-difluorooxaloacetate (K_i = 68 nM). Finally, we propose that cellular uptake of this inhibitor could reduce oxalate production.

Numerous filamentous fungi, including the food biotechnology fungus Aspergillus niger, the opportunistic human pathogen Aspergillus fumigatus, the phytopathogenic fungi Botrytis cinerea and Sclerotinia sclerotiorum, as well as many brown-rot and white-rot basidiomycetes, are able to efficiently produce large quantities of oxalate (1, 2). It is known that oxalate secretion is associated with fungal pathogenesis (1, 3, 4, 5, 6). In the wood-rotting fungus Fomitopsis palustris oxalate is formed as the product of glucose metabolism (7). We recently initiated investigations of the oxalate biosynthetic pathway in order to develop a genomic-based method for distinguishing between oxalate producing and non-producing fungi. An additional goal of this effort was to identify enzyme inhibitors that could be used to arrest oxalate formation in targeted fungi.

In order to attenuate oxalate production in fungi, it is necessary to first identify the major pathway responsible for oxalate formation. There are three potential routes for production of oxalate in fungi: oxidation of glyoxylate (8, 9), oxidation of glycolaldehyde (10), and hydrolysis of oxaloacetate (11). The results of studies of [14C]CO_2 incorporation into the metabolite pools of A. niger indicate that oxalate is derived from oxaloacetate (12). This finding parallels the results of earlier work on the purification of an enzyme "oxalacetalase" (now known as oxaloacetate acetylhydrolase or OAH) that catalyzes the hydrolytic cleavage of oxaloacetate to form acetate and oxalate (11). In a subsequent study, a mutant A. niger strain, NW228 (13), was found to be deficient in both oxalate production and in the

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synthesis of active OAH (14). These observations suggest that oxalate is produced only by the OAH catalyzed process. In the present investigation, we verified the connection between the absence of oxalate formation and the absence of OAH activity in the NW228 mutant by demonstrating that the OAH-encoding oahA gene is interrupted by a stop codon. In an independent effort, Pedersen et al. (15) mutated the A. niger oahA gene by recombination with an oahA sequence-based plasmid, to create a metabolically robust A. niger strain deficient in oxalate production.

OAH isolated from A. niger is reported to be a mixture of N-terminal truncates (16). To obtain homogeneous OAH, we first cloned the A. niger oahA gene for overexpression in E. coli. However, we failed to isolate active transformants. Subsequent efforts focused on cloning the oahA gene from the alternate fungal source Botryis cinerea (previously known as Botryotinia fuckeliana). The cloned B. cinera oahA gene was shown to both restore oxalate production in the A. niger NW228 mutant strain (13, 14) that lacks a functional oahA gene and produce OAH in transformed E. coli cells. In order to provide insight into the mechanism of catalysis and to set the stage for the design of mechanism-based inhibitors, the OAH substrate and metal cofactor specificities were determined. Below we report the results from these studies, which have culminated in the discovery that 3,3-difluorooxaloacetate is a novel tight-binding OAH inhibitor.

MATERIALS AND METHODS

Materials—Oxaloacetic acid, (R)-malic acid, (S)-malic acid, (2R)-methylmalic acid, (2S)-methylmalic acid, (2R, 3S)-isocitric acid were purchased from Sigma-Aldrich. (2R, 3S)-2,3-dimethylmalic acid, (2R)-ethyl-(3S)-methylmalic acid, (2R)-propyl-3S-methylmalic acid, threo-(2R,3S & 2S,3R)-2-methylisocitrate were prepared according to reference (17). (2R, 3S)-isopropylmalate (synthesized by M. Jung) and 3-butylnalate (Aldrich Rare Chemicals, catalog no. S789046) were provided by S. Clarke (University of California, Los Angeles, CA).

3,3-Difluorooxaloacetate—This compound was prepared by modifying the protocol described by Saxy et al (18). A stirred suspension of zinc powder (6.5 g, 0.1 mol) in anhydrous tetrahydrofuran (50 mL) containing Me2SiCl (4 mL, 0.04 mol) was stirred at room temperature for 15 min and then at reflux while ethyl bromodifluoroacetate (24.24g, 0.12mol) was added. To this solution was added ethyl formylformate (5.2 g, 0.05 mol) at a rate sufficient to maintain gentle reflux. After stirring at reflux for 1 h, the solution was cooled to room temperature and concentrated in vacuo. The resulting residue was dissolved in water and then extracted with ethyl acetate. The extracts were washed with water, dried and concentrated in vacuo. The resulting residue was subjected to silica gel column chromatography (methylen chloride and 1:5 ethyl acetate/hexane) to give diethyl 2,2-difluoro-3-hydroxysuccinate (7.9 g, 69%).

To a solution of sulfuric acid (10 mL, 0.18 mol) and pyridinium chlorochromate (0.093 mol) in water (40 mL) was added drop-wise diethyl 2,2-difluoro-3-hydroxysuccinate (7.0 g, 0.031 mol). After stirring at room temperature for 7 h, the mixture was filtered. The filtrate was concentrated in vacuo to give a residue, which was subjected to silica gel column chromatography (10:1 to 3:1 ethyl acetate/hexane) to yield homogeneous diethyl 3,3-difluorooxaloacetate (3.75g, 50%). The spectroscopic data of this substance matched those reported earlier (19).1H NMR (CDCl3) 1.34 (m, 6H), 4.39 (m, 4H); 13C NMR 167.1, 162.3 (t, J = 31.0 Hz), 110.8 (t, J = 263.5 Hz), 91.9 (t, J = 28.1 Hz), 64.1, 63.7, 13.7.

A mixture of diethyl 3,3-difluorooxaloacetate (1.70 g, 7.0 mmol) and 13 mL of 3 M HCl was stirred at reflux for 1.3 h, cooled to room temperature, and concentrated in vacuo. Addition of 2 mL TFA to the residue resulted in the formation of a solid, which was filtered and dried in vacuo to yield 3,3-difluorooxaloacetate (1.18 g, 90%). In agreement with a previous report (19), the 13C NMR (D2O) spectrum is as follows: 169.9, 165.4 (t, J = 29.4 Hz), 112.0 (t, J = 261.1 Hz), 92.0 (t, J = 27.2 Hz).

A. niger OAH Gene Cloning and Sequencing—
The oahA genes from the A. niger wild type strain N400 (CBS120.49), and derived mutant strains NW228 (prtF28) and NW229 (prtF29) (13), were amplified by using standard PCR protocol in conjunction with the forward primer CTGCCCCTTCTTTCTATC and the reverse primer CATCAGAACAGAACAC. The PCR
products were cloned using the vector pGEM®-T Easy (Promega) and sequenced. The nucleotide sequence of the N400 strain has been deposited in the public databases under accession no AJ567910.

Cloning of the B. cinerea oahA Gene—The GenomeWalker kit (clontech laboratories Inc.) was used to obtain the PCR product of the genomic region containing the OAH encoding gene from B. cinerea strain B05.10. The sequences of gene specific primer 1 (ATCAACACAATATCGAGGTTATCG) and primer 2 (GCACGAATTCTCATGT-AGTACTCCTCT) were derived from B. cinerea EST AL117176. The complete oah gene along with 308 bp of the upstream region was amplified, cloned in the vector pGEM®-T Easy (Promega) and sequenced. Standard RT-PCR techniques were used to verify the two introns. The nucleotide sequence has been deposited in the public databases under accession no AY590264.

Complementation of the A. niger prtf Mutation—Three different constructs were made for complementation of the prtf mutation with the B. cinerea oah gene. These are the full length B. cinerea oah gene including the 308 bp upstream region and two fusion constructs in which the A. niger pkiA (20) promoter was fused to the two possible start codons. All three constructs where co-transformed into strain NW188 (pyrA6, prtf28) as previously described (21). Transformants where screened for oxalate production by growing single colonies on complete media (CM) (22) plates with 10 ng/ml methyl orange as pH indicator. Only the transformants in which the pkiA promoter was fused to the most upstream start codon showed oxalate production as verified by HPLC analysis. The copy number of OAH B. cinerea of the transformants was determined by Southern analysis using the pkiA promoter as a radiolabeled probe. The copy number was estimated by comparing the intensity of the native A. niger pki promoter band (1 copy) to the intensity of the band of the introduced pkiA-BcoahA construct.

Targeted Mutagenesis of Botrytis cinerea—A gene replacement construct containing fragments originating from either end of the BcoahA gene flanking a hygromycin resistance cassette (Genbank accession AJ439603) was prepared. The 5′-flanking fragment (466 bp) was amplified using the primers CCCAATCTCAGAAGCAGATC and GATTAAAGATTAATACGCTTCCAGC GGGAAGCAGTGGTAC. The 3′-flanking fragment (611 bp) was amplified using the primers GGGTACGGAGTCGAAATT- CGTGGGACATCTCCAAAGGC and CCAACCAGGTACTGAGATCAG. The flanking fragments were joined to the hygromycin cassette by overlap extension. The PCR mixture contained the three template fragments and the primers GACCTCTAGGTATTTCCGGTG and CTTCTCAAGACCATCCGGCA. The resulting PCR fragment was excised from the gel and directly transformed to B. cinerea protoplasts using the published procedure (23).

Purification of Recombinant Botrytis cinerea OAH from the OAH-pET3c E. coli Clone—The OAH gene was amplified by using a PCR-based strategy (24) with the OAH-pGem-T easy vector clone serving as the template. Pfu DNA polymerase (Stratagene) and oligonucleotide primers containing NdeI and BamHI restriction sites were used for the subcloning the OAH gene into the pET-3c vector (Novagen). The recombinant plasmid, OAH-pET3c, was used to transform competent E. coli BL21(DE3) cells (Novagen). The transformed cells were grown at 20 °C with mild agitation (180 rpm) in Luria broth (LB) containing 50 μg/ml carbenicillin. After 19 h of cell growth (OD600nm ~ 0.7), induction was initiated with 0.4 mM IPTG (RPI Corp.). The culture was incubated for 12.5 h at 20 °C under conditions of vigorous mixing (200 rpm). The cells were harvested by centrifugation (6500 RPM (7,808g)) for 15 min at 4°C in a yield of 4 g/ L of culture media. The cell pellet (23 g) was suspended in 230 ml of ice-cold lysis buffer (50 mM K’ Hapes (pH 7.5), 1 mM EDTA, 1 mM benzamide hydrochloride, 0.05 mg/ml trypsin inhibitor, 1 mM 1, 10-phenanthroline, 0.1 mM PMSF and 5 mM DTT). The suspension was passed through a French Press at 1200 PSI, and then centrifuged at 4 °C for 60 min at 20000 RPM (48,384g). The supernatant was fractionated by ammonium sulfate induced protein precipitation. The 30–40% ammonium sulfate protein precipitate was dialyzed at 4 °C against 4 x 2 L Buffer A (50 mM triethanolamine (pH 7.5), 5 mM MgCl2 and 5 mM DTT) before loading onto a 4.5 x 45 cm DEAE-cellulose column equilibrated with 2 L
Buffer A. The column was washed with 1 L of Buffer A, and then eluted with a 2 L linear gradient of 0 to 0.3 M KCl in Buffer A. The column fractions were analyzed by measuring the absorbance at 280 nm and by carrying out SDSPAGE analysis. Ammonium sulfate was added to the combined fractions to generate 20 % saturation. The resulting solution was loaded onto a 3 x 30 cm Butyl Sepharose column equilibrated at 4 °C with 20 % ammonium sulfate in 500 mL of Buffer A. The column was washed with 450 mL of 20 % ammonium sulfate in Buffer A and then eluted with a 1 L linear gradient of 20 % to 0 % ammonium sulfate in Buffer A. The column fractions were analyzed by measuring the absorbance at 280 nm and by carrying out SDS-PAGE analysis. The OAH eluted at 4 % ammonium sulfate. The OAH-containing fractions were combined, concentrated at 4 °C with an Amicon concentrator (Amicon) and then dialyzed against Buffer A. The resulting sample was concentrated using a MACROSEP 10K OMEGA for storage at −80 °C. The protein sample was shown to be homogeneous by SDS-PAGE analysis. Yield: 4 mg protein/g wet cell.

Recombinant Botrytis cinerea OAH N-Terminal Sequence Determination—OAH was chromatographed on a SDS-PAGE gel, transferred to a PVDF membrane (Novex Co.) and subjected to automated protein N-terminal amino acid sequencing by Dr. Brian M. Martin of the National Institutes of Mental Health (Molecular Structure Unit, Department of Neurotoxicology, Bethesda Maryland, USA) to obtain the sequence PAYSDKVMLT.

Recombinant Botrytis cinerea Molecular Mass Determination—The theoretical subunit molecular mass of recombinant OAH was calculated by using the amino acid composition, derived from the gene sequence, and the EXPASY Molecular Biology Server program Compute pI/MW (25). The subunit size of recombinant OAH was determined by SDS-PAGE analysis with molecular weight standards from Invitrogen. The subunit mass was determined by MS-ES mass spectrometry (University of New Mexico Mass Spectrometry Lab). The molecular weight of native recombinant OAH was first determined using gravity flow gel filtration techniques. The chromatography of OAH was carried out with a 1.5 x 180 cm Sepharose S-200 column (Pharmacia) equilibrated with Buffer B (25 mM K’Hepes, 0.15 M KCl, 0.5 mM DTT, pH 7.5) at 4 °C. The Pharmacia Gel Filtration Calibration Kit (catalase 232,000; aldolase 158,000; albumin 67,000; Ovalbumin 43,000; chymotrypsinogen A 25,000; Ribonuclease A 13,000) was used to calibrate the column according to the manufacturer’s instructions. The chromatography was carried out at 4 °C using Buffer B as eluant and a peristaltic pump to maintain a constant flow rate of 1 mL/min. The plot of the elution volume of the molecular weight standards vs log molecular weight was found to be linear. The OAH molecular weight was thus derived from the measured elution volume by extrapolation. OAH native mass was also analyzed at the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory at Yale University by size exclusion chromatography coupled with on-line laser scattering, refractive index, and ultraviolet detection.

Oxaloacetate Hydrolase Assay—OAH activity was assayed according to a published procedure (11). Reaction solutions (1 mL) contained 0.06-1.0 mM oxaloacetate, 0.032 μM OAH and 5 mM MgCl₂ or 0.18-1.2 mM oxaloacetate, 0.011 μM OAH and 0.3 mM MnCl₂ in 0.1 M imidazole (pH 7.6 and 25 °C). The reaction was monitored at 255 nm for the disappearance of the enol tautomer of oxaloacetate (Δε = 1.1 mM⁻¹ cm⁻¹). The rate of oxaloacetate consumption via spontaneous decarboxylation was measured prior to initiating the enzymatic reaction in order to determine the “background rate”. The background rate was subtracted from the reaction rate measured in the presence of OAH. The influence of the buffer properties on the OAH kinetic behavior was tested by replacing the 0.1 M imidazole of the reaction solutions with 50 mM K’HEPES (pH 7.5) or 50 mM Tris-HCl (pH 7.5).

Assay for Malate Substrates—Continuous Assay. The OAH (13 μM) lyase activity towards 1 mM (R)-malate, (S)-malate, 2S-methylmalate, (2R, 3S)-isocitrate, threeo-(2R,3S & 2S,3R)-2-methylisocitrate, (2R,3S)-isopropylmalate or 3-butyldmalate was measured using 1 mL reaction solutions containing 5 mM MgCl₂, 20 units/mL LDH, and 0.2 mM NADH in 50 mM K’Hepes (pH 7.5 and 25 °C). The absorbance of the solution was monitored at 340 nm (Δε = 6.2 mM⁻¹ cm⁻¹).
The kinetic constants for OAH lysis of (2R)-methylmalate, (2R, 3S)-dimethylmalate, and (2R)-ethyl-(3S)-methylmalate were determined with assay solutions in which the reactant concentration is varied from 0.5Km to 10Km. In the case of 2R-ethyl-3S-methylmalate, reaction solutions contained 600 units of LDH.

**Fixed-Time Assay.** Reaction solutions initially containing 6 μM OAH, 0.50-3.75 mM (2R)-propyl-(3S)-methylmalate, 5 mM MgCl₂, and 50 mM K’Hepes (pH 7.5, 25 °C) were analyzed at ~20 % conversion. A 200 μL aliquot was mixed with 200 μL of 1 N HCl and 100 μL of 0.4 M phenylhydrazine hydrochloride. The solution was stirred for 12 min before the absorbance was measured at 326 nm (2-oxovaleric acid hydrazone molar extinction constant is 6.8 mM⁻¹ cm⁻¹). The control reaction lacked OAH.

**Steady-State Kinetic Constant Determination for Recombinant Botrytis cinerea OAH Substrates**—The steady-state kinetic parameters (Km and kcat) were determined from the initial velocity data measured as a function of substrate concentration. The initial velocity data were fitted to Equation 1 with KinetAsyst 1

\[ V_0 = V_{max}[S]/(K_m + [S]) \]  

(1)

where [S] is the substrate concentration, V₀ is the initial velocity, Vmax is the maximum velocity, and Kᵢₘ is the Michaelis-Menten constant for the substrate. The kcat value was calculated from Vmax and the enzyme concentration using the equation 

\[ k_{cat} = V_{max}[E], \]  

where [E] is the protein subunit molar concentration in the reaction calculated from the ratio of measured protein concentration and the protein molecular mass (34486 Da).

**Determination of Inhibition Constants for Recombinant Botrytis cinerea OAH Inhibitors**—For determination of the competitive inhibition constant Ki the 1 mL reaction solutions initially contained 0.1 M imidazole (pH 7.6), 5 mM MgCl₂, 9 nM oxaloacetate hydrolase, varying concentrations of oxaloacetate (0.04-1.0 mM) and changing, fixed concentrations of difluorooxaloacetate (0, 0.2, 0.4 μM), oxalate (0, 25, 50 and 100 μM), (R)-malate (0,10 and 20 mM) or (S)-malate (0, 20 and 40 mM). Initial velocity data were fitted to equation 2.

\[ V_0 = V_{max} \left[ S/((K_{m1}+V/K_{i})) + [S] \right] \]  

(2)

**Metal Ion Activation of Recombinant Botrytis cinerea OAH**—The metal ion-free protein was prepared by dialysis against 7 changes of 500 mL of 30 mM EDTA/50 mM triethanolamine/5mM DTT (pH 7.5; 4 °C) followed by dialysis against 6 changes of 500 mL of 50 mM triethanolamine/5 mM DTT (pH 7.5, 4 °C). The initial velocity of oxaloacetate consumption in reaction solutions containing OAH, 1 mM oxaloacetate, and varying concentrations of MgCl₂, MnCl₂, CoCl₂, CaCl₂, ZnCl₂, FeSO₄, CuBr₂, NiCl₂ in 0.1 M imidazole (pH 7.6), 50 mM K’HEPES (pH 7.5) or 50 mM Tris-HCl (pH 7.5) at 25°C. The initial velocity data were analyzed using Equation 1 and the computer program KinetAsyst 1.

**RESULTS**

**Cloning and sequencing of the oahA gene from oxalate non-producing mutant strains**—The oahA genes from the A. niger mutant strains, prtf28 (NW228) and prtf29 (NW229) were sequenced and their sequences were compared to that of the oahA gene contained in the wild-type parental strain. Previous studies had shown the mutant strains lack both oxalate production and OAH activity (14, 26). However it remained to be demonstrated that the oahA gene is disrupted. The sequence analysis verified that the oahA genes from the mutant strains had acquired a stop codon in the open reading frame (ORF) (at amino acid position R94 (NW228) or Q159 (NW229)). Furthermore, by using a pH indicator plate assay (Figure 1) and HPLC analyses (data not shown) we demonstrated that the mutant strains, transformed with the wild type oahA gene, regain their oxalate production capability.

**Cloning and disruption of the Botrytis cinerea oahA gene**—The results from the complementation experiment described above strongly suggest that oxalate production in A. niger is caused solely by OAH catalyzed cleavage of oxaloacetate. We scrutinized the genomes of other fungal oxalate producers to see if they too encoded OAH. BLAST searches using the A. niger oahA gene (AnoahA) as the query suggested that EST AL1117176 from the oxalate-producer Botrytis cinerea represents a partial copy of the B. cinerea OAH encoding gene. The complete BcoahA gene, isolated from the B. cinerea genome by using PCR and genome walking techniques, was then used for complementation of the prtf28
mutation. The sequence of the putative BcOAH was shown to be 70% identical to that of the A. niger OAH. Because there are two possible start codons separated by 7 amino acids, three different constructs were made for complementation purposes. These are the full length B. cinerea oahA gene (including the 308 bp upstream region calculated from the most upstream candidate start codon) and two fusion constructs, in which the constitutive A. niger pkiA promoter (20) is fused to one of the two possible start codons. Complementation of the A. niger NW228 non-oxalate producing strain was achieved with the BcoahA coding region that included the most upstream start codon fused to the strong A. niger pkiA promoter (20). Seven independent pkiA::BcoahA transformants were identified by using an indicator plate assay (Figure 2). The integration of the construct within the transformants was verified by using Southern analysis (Figure 3). HPLC analysis was used to demonstrate that each transformant had regained the ability to produce oxalate (data not shown).

To confirm the role played by the BcoahA gene in oxalate biosynthesis in B. cinerea, the gene was deleted from the B. cinerea genome by employing targeted gene replacement. Transformants, in which homologous recombination had occurred, were identified by PCR screening and by Southern blot analysis. Five independent transformants were identified as having perfect gene replacement in the absence of additional ectopic integration (data not shown). The pH indicator plate assay (Figure 2) was used to demonstrate that the BcoahA-deficient mutants do not produce a detectable level of oxalate.

**Purification of Recombinant B. cinerea OAH and Size Determination**—The DNA sequence of the subcloned gene was found to agree with the published sequence (GenBankTM accession number AY590264) except that the nucleotide at position 1637 is G not A. Thus, the encoded amino acid is Ala not Thr. The recombinant OAH was purified to homogeneity in an overall yield of 4 mg/g wet cells by using the 4-step protocol summarized in Table 1. The N-terminal sequence of OAH revealed that the N-terminal methionine is lost during post-translational modification. This was confirmed by a molecular mass determination by using mass spectroscopy. The theoretical mass of OAH-Met is 34355 Da compared to the experimental value of 34355 ± 1 Da. The SDS-PAGE analysis gave an estimated subunit mass of 35 kDa, whereas the native mass measured by using molecular size gel filtration chromatography is ~100 kDa. This result is indicative of a homotrimeric quaternary structure. The α,β-barrels of the enzymes of the PEP mutase/isocitrate lyase superfamily incorporate the C-terminal α-helix from an adjacent subunit, a family structural trait known as ‘helix swapping’. The functional unit is therefore a dimer, and the quaternary structure that has been most frequently encountered is the homotetramer ((17) and references therein). Because a trimeric structure is not consistent with this pattern, we examined the possibility that the association state of OAH is unstable. Native molecular mass determination, by using size exclusion chromatography, coupled with on-line laser scattering, refractive index, and ultraviolet detection provided additional information regarding the OAH quaternary structure. A sample of OAH eluted in a single peak from size exclusion fractionation was polydispersed with a MW range of 50 kDa at 0.05 mg/ml to 114 kDa at 0.3 mg/mL and averaged MW of 97 kDa. At 1.1 mg/ml the sample reached an averaged MW of 118 Da and a hydrodynamic radius of 4.1 nm. The hydrodynamic radius remained at 4.1 nm at 3 mg/ml indicating that the protein does not exist in oligomeric forms higher than a tetramer. The results are consistent with the existence of a monomer-dimer-tetramer equilibrium.

**Metal Ion Specificity of B. cinerea OAH**—Enzymes of the isocitrate lyase/PEP mutase superfamily require a divalent metal ion for catalysis. The steady-state kinetic constants for metal ion activation of OAH were determined at a saturating concentration of oxaloacetate (1 mM) and varying metal ion concentration using 0.1 M imidazole (pH 7.6), 50 mM K’Hepes (pH 7.5) or 50 mM Tris-HCl (pH 7.5) as buffer. The $k_{cat}$ and $K_m$ values, measured for the Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$ activation are listed in Table 2. Mn$^{2+}$ and Mg$^{2+}$ are significantly better activators for OAH than is Ca$^{2+}$. The $k_{cat}$ values determined using the 0.1 M imidazole buffer do not differ significantly from those measured using the K’Hepes buffer. The $K_m$ values measured using the K’Hepes buffer (which does not bind divalent metal ions) are slightly smaller than those measured using the imidazole buffer.
buffer (which does bind metal ions). OAH is not subject to metal ion inhibition by Mg\(^{2+}\) (no inhibition observed at 5 mM MgCl\(_2\)). Likewise, inhibition was not observed at 0.3 mM MnCl\(_2\). The metal ions Zn\(^{2+}\) (100 μM), Co\(^{2+}\) (300 μM), Fe\(^{2+}\) (100 μM), Cu\(^{2+}\) (500 μM) and Ni\(^{2+}\) (1 mM) failed to activate OAH.

**Substrate Specificity and Kinetic Properties of B. cinerea OAH**—The steady-state kinetic constants for OAH catalyzed hydrolysis of oxaloacetate were measured as a function of oxaloacetate concentration at fixed metal ion cofactor concentration (5 mM MgCl\(_2\) or 0.3 mM MnCl\(_2\)). Reactions were carried out at 25 °C using 0.1 M imidazole (pH 7.6), 50 mM K Hepes (pH 7.5) or 50 mM Tris-HCl (pH 7.5) as buffer. The results are summarized in Table 2. Mn\(^{2+}\) activated OAH displays a higher turnover rate than does the Mg\(^{2+}\) activated OAH. On the other hand, the K\(_{m}\) of oxaloacetate measured by using Mn\(^{2+}\) as activator is larger than that measured with Mg\(^{2+}\) serving as the activator. Consequently, there is no significant difference in the specificity constant k\(_{cat}\)/K\(_{m}\) between Mg\(^{2+}\) and the Mn\(^{2+}\) activated OAH.

The ability of OAH to catalyze C-C bond cleavage in α-hydroxyoxycarboxylate metabolites was probed in order to determine if OAH retains the C-C lyase activity characteristics of the C-C bond lyase branch of the isocitrate lyase/PEP mutase family (viz. petal death protein, isocitrate lyase and 2-methylisocitrate lyase) (17) and references therein). The results are listed in Table 3. The first set of substrates tested are the R- and S-enantiomers of malate. To enhance the sensitivity for detection of product formation, reaction mixtures containing high concentrations of OAH (13 μM) were used. In addition, to ensure saturation of catalytic sites high concentrations (1 mM) of the reactants were employed. Under these conditions, no C-C lyase activity is observed, suggesting that the k\(_{cat}\) for cleavage of malate is less than the detection limit which is 1×10\(^{-5}\) s\(^{-1}\).

Next, substrate activities of C(2) alkyl malates were probed. Whereas the (2S)-methylmalate is not a substrate for OAH, the (2R)-methylmalate is converted to pyruvate and acetate with a k\(_{cat}\) = 0.01 s\(^{-1}\) and a K\(_{m}\) = 1.45 mM by this enzyme. The addition of a methyl group at the C(3) position of the (2R)-methylmalate leads to a further improvement in substrate activity. The K\(_{m}\) value of (2R, 3S)-dimethylmalate is 10-fold smaller than that for (2R)-methylmalate. However, the k\(_{cat}\) values measured for these two substrates are equivalent. The substrate (2R)-ethyl-(3S)-methylmalate displayed a 10-fold larger k\(_{cat}\) and a comparably larger K\(_{m}\). The k\(_{cat}\)/K\(_{m}\) value measured for (2R)-propyl-(3S)-methylmalate is significantly smaller (49 M\(^{-1}\)s\(^{-1}\)), a finding that suggests that the active site has limited space for the C(2) alkyl group.

The native substrates of isocitrate lyase ((2R, 3S)-isocitrate) and 2-methylisocitrate lyase ((2R, 3S & 2S,3R)-2-methylisocitrate) are not substrates for OAH. This observation indicates that the OAH active site cannot accommodate a C(3) CH\(_2\)COO substituent.

In summary, (2R, 3S)-2,3-dimethylmalate and (2R)-ethyl-(3S)-methylmalate are the most active of the malate substrates tested, each having a k\(_{cat}\)/K\(_{m}\) of ca. 2×10\(^{2}\) M\(^{-1}\)s\(^{-1}\). The petal death protein is known (17) to possess the same substrate specificity: oxaloacetate (k\(_{cat}\)/K\(_{m}\) = 2×10\(^{4}\) M\(^{-1}\)s\(^{-1}\)) and (2R)-ethyl-(3S)-methylmalate (k\(_{cat}\)/K\(_{m}\) = 3×10\(^{3}\) M\(^{-1}\)s\(^{-1}\)) > (2R)-propyl-(3S)-methylmalate (k\(_{cat}\)/K\(_{m}\) = 3×10\(^{3}\) M\(^{-1}\)s\(^{-1}\)) > (2R)-methylmalate (k\(_{cat}\)/K\(_{m}\) = 7×10\(^{2}\) M\(^{-1}\)s\(^{-1}\)) >> (R)-malate and isocitrate. However, the catalytic efficiencies for cleavage of these substrates by the petal death protein are considerably higher than that observed for OAH.

**Inhibition of B. cinerea OAH**—The competitive inhibition constants of oxalate, (R)-malate, (S)-malate and 3,3-difluorooxaloacetate were evaluated in order to gain information about the structural determinants for ligand binding. Oxalate, a product of oxaloacetate cleavage, binds to OAH with high affinity (K\(_{i}\) = 19 ± 1 μM). Oxalate is also an analog of the pyruvate enolate anion intermediate formed in the cleavage of (2R, 3S)-2,3-dimethylmalate. The petal death protein binds oxalate tightly (K\(_{i}\) = 4.3 ± 0.3 μM), as does isocitrate lyase and PEP mutase (17).

3,3-Difluorooxaloacetate, which differs from oxaloacetate by replacement of the (3) hydrogens with fluorine atoms, is not a substrate for OAH (the k\(_{cat}\) detection limit is 1×10\(^{-5}\) s\(^{-1}\)). However, this substance is an exceptionally tight binding linear competitive inhibitor: K\(_{i}\) = 68 ± 4 nM) (Figure 4). In contrast, malate is a weak binding competitive inhibitor of OAH. Malate differs from
the oxaloacetate in that its C-2 center is tetrahedral and functionalized with a hydroxyl group. (R)-Malate (K_i = 2.5 ± 0.2 mM) binds an order of magnitude tighter than does its S-enantiomer (K_i = 22 ± 3 mM), but ~5-orders of magnitude less tightly than does 3,3-difluorooxaloacetate.

DISCUSSION

**OAH Function and Distribution**—The observations described above show that (1) oxalate formation in the fungi *A. niger* and *B. cinerea* derives from OAH catalyzed hydrolytic cleavage of oxaloacetate, and (2) the respective genomes of *A. niger* and *B. cinerea* contain a single OAH encoding gene. Although, the two OAhs share 70 % sequence identity, they display different behavior. The *A. niger* OAH is reported to associate to form high order oligomers and to be specific for Mn^{2+} as the metal ion cofactor. Both enzymes do, however conserve the active serine residue (Ser260 of *B.cinerea*) known to contribute significantly to OAH catalytic efficiency (27).

BLAST searches, in which the *A. niger* OAH sequence is used as query, have led to the identification of one or more close homologs (as defined by > 50 % sequence identity) encoded in the *A. niger* genome, and in the genomes of other *Aspergillus* strains. The homologs of *A. niger* do not posses the active site serine found in authentic OAH. Also, inactivation of the OAH encoding gene results in loss of oxalate formation. Presently, their catalytic functions are unknown.

*A. niger* and *B. cinerea* are representatives of different subphyla of *ascomycete* fungi, separated by hundreds of millions of years of evolution. Our results strongly suggest that both fungi use OAH catalyzed hydrolytic cleavage of oxaloacetate as the main (if not the sole) route for oxalate production. Among the 22 fungi, whose genomes have been sequenced, the presence of the *oah* gene is strictly correlated with oxalate production (27). The acquisition of OAH activity in a given fungal species for oxalate production appears to be important for niche adaptation.

We searched for evidence of the occurrence of OAH in organisms from other kingdoms. Earlier reports indicate that oxalate biosynthesis occurs in some plants, especially in plants of the genus *oxalis* (17, 28, 29, 30, 31). Studies carried out with plant tissue extracts indicate that enzyme catalyzed conversion of oxaloacetate to oxalate does take place (30). However, owing to the scarcity of sequenced plant genomes, plant *oah* genes have not yet been identified. One exception is the gene encoding the petal death protein of the flowering plant *Dianthus caryophyllus* (Swiss-Prot entry Q05957). The petal death protein possesses OAH activity in addition to 2-alkylmalate C-C bond lyase activity (17a). A BLAST search of the gene data bank, identified homologs of ~60 % sequence identity in the plants *Arabidopsis thaliana* and *Oryza sativa*. However, neither of these homologs have been isolated or tested for activity and neither appear to possess an active site Ser which is well correlated with the presence of OAH activity (27).

The presence of OAH activity has been observed in extracts of the bacterium *Streptomyces cattleya* (32). Unfortunately, the sequence of the gene associated with this activity has not been reported. To identify other bacterial proteins that might possess OAH activity, we carried out a BLAST search of bacterial genomes using the *D. caryophyllus* petal death protein and the *B. cinerea* OAH as query. The *Bacillus cereus* Swiss-Prot entry Q738L6 was found to have the highest homology. This protein is similar in size (viz 302 amino acids) to the petal death protein and the *B. cinerea* OAH. Furthermore, it shares 39 % sequence identity with the petal death protein and 36% identity with the *B. cinerea* OAH. However, it does not appear that the *B. cereus* protein contains the conserved active site serine associated with OAH activity (27). Rather, this protein has a proline residue at this position as do the other bacterial homologs of >35% sequence identity reported to date. The recombinant *B. cereus* Q738L6 was prepared by gene cloning and expression in *E. coli* and the purified enzyme was subjected to substrate screening (33). The *B. cereus* Q738L6 was found to specifically catalyze the cleavage of 2-methylisocitrate to form succinate and pyruvate (k_{cat}/K_m = 2.5 × 10^6 M^{-1} s^{-1}) (33). Thus, the bacterial homolog is not OAH but instead 2-methylisocitrate lyase of the propionate pathway.

Based on the results of this survey we conclude that OAH is widely used among fungi for the purpose of oxalate formation associated with niche adaptation. Moreover, OAH may also function in oxalate producing plants and perhaps in some specialized bacteria.
**OAH Catalysis, Inhibition and Evolution**—OAH catalyzes the hydrolytic cleavage of oxaloacetate (a retro-Claisen reaction) with high efficiency ($k_{cat}/K_m = 1.5 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) (Table 2). In addition, it promotes retro-Aldol reactions of 2R,3S-dimethylmalate and 2R-ethyl-3S-methylmalate with a 1000-fold lower efficiency ($k_{cat}/K_m = 2 \times 10^2 \text{ M}^{-1} \text{s}^{-1}$) (Table 3). The plant petal death protein displays these same types of catalytic activities but it promotes both processes with equal catalytic efficiency: $k_{cat}/K_m = 2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ for hydrolytic cleavage of oxaloacetate and for lysis of 2R-ethyl-3S-methylmalate (17).

OAH and the petal death protein are members of the PEP mutase/isocitrate lyase enzyme superfamily (17, 34-40). Together with isocitrate lyase and 2-methylisocitrate lyase, they form a subgroup of enzymes of similar chemical function. Within this subgroup, the superfamily catalytic scaffold is tailored to bind and activate α-oxocarboxylate metabolites for Coα-Cβ bond cleavage (17, 35-39). The ability of OAH and the petal death protein to act on C(α)OH and C(α)=O substrates is quite remarkable. This dual catalytic activity might also be possible for isocitrate lyase, but to our knowledge this has not been tested.

The catalytic mechanism that has been proposed for the isocitrater and 2-methylisocitrate lyases is depicted in Figure 5 (39). The key elements of catalysis are (1) electron withdrawal from the C(α)oxyen via electrostatic interaction with the Mg$^{2+}$ cofactor and the conserved Arg residue, (2) electron withdrawal from the aci-carboxylate leaving group via hydrogen bond formation with the conserved glutamic acid residue, (3) general acid catalysis by the conserved Cys residue, and (4) general base catalysis by a yet to be assigned residue.

These elements are conserved in the petal death protein for which the active site structure is known (17b). Although the X-ray structure of OAH is not yet available, it is evident from sequence alignment analysis that these same elements contribute to the OAH catalysis. Thus, the catalytic mechanism proposed for the isocitrater and 2-methylisocitrate lyases could be operative in the petal death protein and OAH mediated cleavage reactions of the 2R-alkylmalates. It is tempting to assume that the OAH catalytic scaffold is designed to promote reaction by way of a single catalytic mechanism (Figure 5, pathway 1). If so, the only adjustment required for operation of a retro-Aldol type mechanism would be the hydration of oxaloacetate to generate the C(α) gem-diol. The C(α) gem-diol is expected to bind in the active site in the same manner as do 2R-alkylmalates with the pro-S OH of the gem-diol located in place of the C(α) alkyl groups of the malates. Modeling studies indicate that Ser257 in the petal death protein is properly positioned for interaction with the pro-S OH of the oxaloacetate hydrate (27). The corresponding residue in OAH is Ser260.

Although the economy of this hydrate-retro-Aldol type mechanism is noteworthy, proof of its operation is needed. In theory, the OAH catalyzed oxaloacetate cleavage reaction might proceed by any one of several reasonable chemical pathways (Figure 5, pathways 1-4), each of which is distinguished by the form of the oxaloacetate that serves as substrate. In aqueous solution at neutral pH, the keto-form of oxaloacetate (81%) exists in equilibrium with the hydrate form (i.e., the gem-diol) (7%) and the enol form (12%) (41). Oxaloacetate binds Mg$^{2+}$ (and Mn$^{2+}$) (42). Consequently the ratio of the structural forms at equilibrium is changed, and owing to the reduction in $pK_a$, the enolate form may also contribute to this equilibrium (42). At the current time, it is not clear whether the “free” enzyme binds the Mg$^{2+}$(oxaloacetate) complex or the enzyme-Mg$^{2+}$ complex binds the “free” oxaloacetate. Furthermore, the structural form of the oxaloacetate (ketone, gem-diol, enol or enolate) that is bound in the enzyme active site is not known. Therefore, each of the 4 chemical pathways depicted in Figure 5B must be considered.

Whereas the gem-diol is a minor component of solvated oxaloacetate, it is the major component by far of solvated 3,3-difluorooxaloacetate. In the latter case, only the gem-diol is observed by the $^{13}$C-NMR. However, the report that aspartate transaminase slowly catalyzes the conversion of difluorooxalacetate to difluoropropionate (43) suggests that the ketone form is present, if only in a trace amount. The high affinity exhibited by OAH for the competitive inhibitor 3,3-difluorooxaloacetate ($K_i = 68$ nM; Figure 4) suggests that the enzyme preferentially binds the gem-diol form of oxaloacetate. Importantly, the
gem-diol of oxaloacetate serves as a substrate in pathway 1 (Figure 5B), and as an intermediate in pathways 2 and 3. Only in pathway 4, involving the oxaloacetate enolate, does the gem-diol not serve as an intermediate. Interestingly, the electrostatic forces that are needed to stabilize the oxynion intermediate formed in pathway 4, are not required to bind the gem-diol intermediate in pathways 1-3. The results of earlier isotope labeling studies, carried out with the OAH from A. niger, provide evidence that catalysis by this enzyme does not take place through the enol (or enolate) (44). Specifically, the acetate derived from OAH catalyzed conversion of the oxaloacetate generated in situ by malate dehydrogenase catalysis of S-malate uniformly labeled with $^{14}$C in mixture with S-malate doubly labeled with tritium at C(2), was found to have a $^3$H/$^{14}$C ratio twice that of the starting S-malate. Enolization of the oxaloacetate would result in loss of one tritium and the formation of acetate that has the same $^3$H/$^{14}$C ratio as the starting malate. Thus, it appears that pathways 3 and 4 are not followed in the catalytic process.

Based on the fact that pathway 1 is followed in catalysis of the C(α)-C(β) bond cleavage reactions of 2R-alkylmalates and that pathway 2, requires that the enzyme binds and activates a water molecule for nucleophilic attack at the C(α)=O, we propose that the gem-diol retro-Aldol pathway is the most reasonable mechanism for the OAH catalyzed transformation of oxaloacetate to pyruvate and acetate. However, detailed studies are required to provide definitive proof for this mechanism.

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FOOTNOTES

1The abbreviations used are: OAH, oxaloacetate acetylhydrolase; PEP, phosphoenolpyruvate; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis PCR, polymerase chain reaction; K‘Hepes, potassium salt of N-(2-hydroxyethyl)piperazine-N‘-2-ethanesulfonic acid; LDH, lactate dehydrogenase; DTT, dithiothreitol; NADH, nicotinamide adenine dinucleotide; HPLC, high performance liquid chromatography.

FIGURE LEGENDS

Fig. 1. Screen for oxalate production in A. niger using a methyl orange pH-indicator plate assay. The pink color indicates acidification caused by hydrolytic cleavage of oxaloacetate, which produces oxalate, acetate and a hydrogen ion. Panel A: wild type A. niger. Panel B: oxalate non-producing mutant NW228. Panel C: NW228 transformed with the wild type A. niger oahA gene. Panel D: NW228 transformed with the B. cinerea oahA gene.

Fig. 2. Screen for oxalate production B. cinerea using a bromothymol blue pH-indicator plate assay. The yellow color indicates acidification caused by the hydrolytic cleavage of oxaloacetate to oxalate, acetate and a hydrogen ion. Panel A: wild type B. cinerea. Panel B: oahA deletion strain.

Fig. 3. Molecular characterization of A. niger NW228 transformants complemented with pkiA::BcoahA construct. Lane 1 and 2 NW228 control. Lane 3–9: 8 independent NW228 pkiA::BcoahA transformants. Lane 10: Wild type control. The bands corresponding with the native pkiA gene and the construct are indicated.

Fig. 4. The double reciprocal plot of the initial velocity of OAH catalyzed hydrolytic cleavage of oxaloacetate vs the concentration of oxaloacetate (0.04- 1.0 mM) in 0.1 M imidazole/5 mM MgCl₂ (pH=7.6, 25°C) at changing, fixed concentrations of difluorooxaloacetate (circle: 0 nM; filled square: 200 nM; open square: 400 nM). See Materials and Methods for details.

Fig. 5. A. The catalytic mechanism of the α-hydroxycarboxylate lyases of the PEP mutase/isocitrate lyase enzyme superfamily. B. The four pathways leading from oxaloacetate to oxalate and acetate.
Table 1

Experimental protocol for purification of *Botrytis cinerea* OAH from *E. coli* BL21(DE3) cells transformed with the OAH-pET3c clone

| Purification step     | Total protein (g) | Total activity<sup>a</sup> (units) | Specific activity (units/mg) | Yield (%) | Purification (-fold) |
|-----------------------|-------------------|----------------------------------|-----------------------------|-----------|----------------------|
| Cell extract          | 20                | 12300                            | 0.62                        | 100       |                      |
| Ammonium Sulfate      | 2                 | 11300                            | 4.6                         | 92        | 7                    |
| DEAE-Cellulose        | 0.35              | 5300                             | 15                          | 43        | 25                   |
| Butyl-Sepharose       | 0.17              | 3430                             | 20                          | 28        | 33                   |

<sup>a</sup> One unit of enzyme activity is defined as the amount of enzyme required for the consumption of 1 μmol oxaloacetate/min in 0.1M imidazole, 5 mM MgCl₂, and 1 mM oxaloacetate at 25°C and pH 7.6.
Table 2
Steady-state kinetic constants for M$^{12}$ activated OAH catalyzed hydrolytic cleavage of oxaloacetate at 25°C. Reaction solutions were buffered with 0.1 M imidazole (pH 7.6), 50 mM K$^{+}$HEPES (pH 7.5) or 50 mM Tris-HCl (pH 7.5). Kinetic constants reported in parenthesis represent an independent experimental determination.

| Cofactor | Buffer | $k_{cat}$ (s$^{-1}$) | $^{a}K_{m}$ (µM) OAH | $^{b}K_{s}$ (µM) M$^{12}$ |
|----------|--------|---------------------|----------------------|-----------------------|
| Mg$^{12}$ | Imidazole | 10.3 ± 0.4 | 62 ± 5 | |
|          | Hepes   | 9.6 ± 0.3 | 64 ± 6 | |
|          | Tris     | 9.5 ± 0.4 | 84 ± 5 | |
| Mg$^{12}$ | Imidazole | 11.8 ± 0.3 | | 76 ± 4 |
|          | Hepes   | 12.5 ± 0.3 | | 43 ± 3 |
| Mn$^{12}$ | Imidazole | 50 ± 1 | | 240 ± 10 |
| Mn$^{12}$ | Imidazole | 37 ± 1 | | 35 ± 3 |
|          | Hepes   | 32 ± 1 | | 24 ± 3 |
| Ca$^{12}$ | Imidazole | 0.35 ± 0.01 | | 730 ± 90 |

$^{a}$ Reaction solutions contained 0.5-10 x $K_{m}$ oxaloacetate and 5 mM Mg$^{+}$ or 0.3 mM Mn$^{12}$.

$^{b}$ Reaction solutions contained 1 mM oxaloacetate and 0.5-10 x $K_{s}$ M$^{12}$. 
Table 3
Steady-State Kinetic Constants Determined for the C-C Bond Cleavage Reactions Catalyzed by Botrytis cinerea OAH in 5 mM MgCl₂ and 50 mM K²Hepes buffer (pH 7.5 and 25°C). Chemical structures are shown in Chart 1.

| Reactant                        | kₜₜ (s⁻¹) | Kᵣ (µM)  | kₜₜ/Kᵣ (M⁻¹s⁻¹) |
|---------------------------------|-----------|----------|------------------|
| Oxaloacetate                    | 9.9 ± 0.3 | 65 ± 6   | 1.5 x 10⁻² (a)   |
| 2R-methylmalate                 | (1.13 ± 0.02) x 10⁻² | 1450 ± 60 | 7.8 (b)         |
| 2R, 3S-dimethylmalate           | (2.93 ± 0.07) x 10⁻² | 140 ± 10 | 2.1 x 10⁻² (b)  |
| 2R-ethyl-3S-methylmalate        | (3.46 ± 0.09) x 10⁻¹ | 2000 ± 100 | 1.7 x 10⁻² (c) |
| 2R-propyl-3S-methylmalate       | (3.77 ± 0.07) x 10⁻² | 770 ± 40 | 49 (d)          |
| (2R, 3S)-isocitrate             | <10⁻⁵ (b) |          |                  |
| (2R, 3S & 2S, 3R)-2-methylisocitrate | <10⁻⁵ (b) |          |                  |
| R-malate or S-malate            | <10⁻⁵ (b) |          |                  |
| 2S-methylmalate                 | <10⁻⁵ (b) |          |                  |
| 3-isopropylmalate               | <10⁻⁵ (b) |          |                  |
| 3-butylmalate                   | <10⁻⁵ (b) |          |                  |

(a) The kinetic constants were determined using direct optical method.
(b) The kinetic constants were determined using LDH/NADH coupling assay (20 units/mL LDH).
(c) The kinetic constants were determined by LDH/NADH coupling assay (600 units/mL LDH).
(d) The kinetic constants were determined using the fixed-time phenylhydrazine-based assay.
Chart 1

- **R-malate**
  
  ![R-malate structure](attachment:image.png)

- **S-malate**
  
  ![S-malate structure](attachment:image.png)

- **R-2-methylmalate**
  
  ![R-2-methylmalate structure](attachment:image.png)

- **S-2-methylmalate**
  
  ![S-2-methylmalate structure](attachment:image.png)

- **(2R,3S)-2,3-dimethylmalate**
  
  ![2R,3S)-2,3-dimethylmalate structure](attachment:image.png)

- **(2R,3S)-2-ethyl,3-methyl-malate**
  
  ![2R,3S)-2-ethyl,3-methyl-malate structure](attachment:image.png)

- **oxaloacetate**
  
  ![oxaloacetate structure](attachment:image.png)

- **oxaloacetate hydrate (C(2)gem-diol)**
  
  ![oxaloacetate hydrate (C(2)gem-diol) structure](attachment:image.png)

- **difluorooxaloacetate (ketone)**
  
  ![difluorooxaloacetate (ketone) structure](attachment:image.png)

- **difluorooxaloacetate hydrate (C(2)gem-diol)**
  
  ![difluorooxaloacetate hydrate (C(2)gem-diol) structure](attachment:image.png)

- **2R, 3S-isocitrate R=H**
  
  ![2R, 3S-isocitrate R=H structure](attachment:image.png)

- **2R, 3S-methyl-isocitrate R = CH₃**
  
  ![2R, 3S-methyl-isocitrate R = CH₃ structure](attachment:image.png)
Figure 1
Figure 3

- Native pKi
- pkiA::BcoahA
Figure 4

![Graph showing the relationship between 1/[OA] (1/mM) and 1/V (s/μM). The graph includes a linear regression line and data points.]
Figure 5

A

B

Figure 5
Oxaloacetate hydrolase: The C-C bond lyase of oxalate secreting fungi
Ying Han, Henk-Jan Joosten, Weiling Niu, Zhiming Zhao, Patrick S. Mariano, M T. McCalman, J.A.L. van Kan, Peter J. Schaap and Debra Dunaway-Mariano

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