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QM/MM investigation of the reaction rates of substrates of 2,3-dimethylmalate lyase: A catabolic protein isolated from *Aspergillus niger*

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ABSTRACT

Aspergillus niger is an industrially important microorganism used in the production of citric acid. It is a common cause of food spoilage and represents a health issue for patients with compromised immune systems. Recent studies on *Aspergillus niger* have revealed details on the isocitrate lyase (ICL) superfamily and its role in catabolism, including (2*R*, 3*S*)-dimethylmalate lyase (DMML). Members of this and related lyase super families are of considerable interest as potential treatments for bacterial and fungal infections, including Tuberculosis. In our efforts to better understand this class of protein, we investigate the catalytic mechanism of DMML, studying five different substrates and two different active site metals configurations using molecular dynamics (MD) and hybrid quantum mechanics/molecular mechanics (QM/MM) calculations. We show that the predicted barriers to reaction for the substrates show good agreement with the experimental k_{cat} values. This results help to confirm the validity of the proposed mechanism and open up the possibility of developing novel mechanism based inhibitors specifically for this target.

1. Introduction

Aspergillus niger is a fungal species found throughout the world. It is a common cause of food spoilage, particularly in tropical climates [1,2]. It is also a cause of medical concern as certain species produce toxins and/or carcinogens [3]. While infections in healthy animals and human are generally not considered problematic, infections in immuno-compromised patients is a major concern. *Aspergillus niger* is of considerable importance industrially, being used in the production of citric acid since the early 1900s, and the production of certain enzymes since the 1960s [3]. While fungal infections can be treated with broad spectrum antibiotics such as penicillin, there is interest in the development of cheaper alternatives, particularly for the food storage and processing industries to treat *Aspergillus* infections [3–5].

Recent studies on *Aspergillus niger* have revealed extensive biochemical and structural details of the isocitrate lyase (ICL) superfamily and its role in catabolism [6–12]. Members of this super family include ICL [13] itself, oxaloacetate acetyl hydrolase (OAH) [14], 2-methylisocitrate lyase (MICL), petal death protein (PDP) [15] and (2*R*, 3*S*)-dimethylmalate lyase (DMML) [6]. ICL inhibitors have been shown to can inhibit Tuberculosis [16,17], bacterial [18] and fungal infections [18]. Greater knowledge regarding the structure, reactivity and active site selectivity of these targets would be highly valuable in the development of novel inhibitors for this important biological pathway [19]. For example, 3-bromopyruvate has been shown to act as a covalent inhibitor in MICL [20], reacting with the conserved cysteine residue found in all ICL superfamily members [21]. The application of computational methods that can both describe the protein selectivity and the chemical reactivity of the substrate could therefore prove valuable in the development of novel, selective inhibitors [19].

The extensive work of Herzberg and co-workers has shed considerable light on the catalytic mechanism of the ICL superfamily, and in particular DMML [6,7]. The family adopts a distorted α/β barrel tertiary structure in which only 7 of the 8 helices pack up against the 8 stranded parallel β-sheets. The active site structures obtained contain either Mg^{2+} or Mn^{2+} cofactors in an octahedral configuration with 3 water molecules, the side chain of an Asp residue and the bound oxy acid ligand through two interactions. It was origi-

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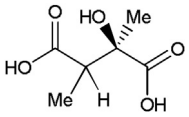
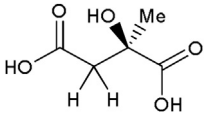
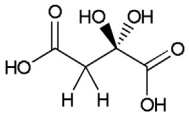
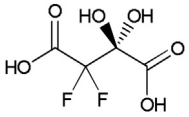
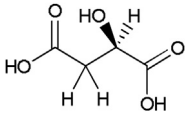
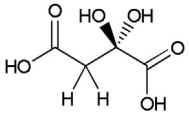
Table 1
Kinetic, thermodynamic and calculated rate determining barriers (kcal/mol) for the DMML substrates investigated. Experimental k_{cat} data taken from reference [52].

Metal	ID	Structure	k_{cat} (s^{-1})	ΔE Barrier	ΔE SP Barrier	ΔG Barrier
Mn^{2+}	*(2R, 3S)*-Dimethylmalate (1)		19.2	10.6	7.4	9.6
	(2R)-Methylmalate (2)		2.6	11.4	8.6	11.9
	Oxaloacetate (3)[a]		0.48/0.92	8.1	7.3	9.9
	3,3-Difluoro-oxaloacetate (4)[b]		$<1 \times 10^{-5}$	19.8	16.8	20.7
	S-Malate (5)		$<1 \times 10^{-5}$	17.2	17.3	15.3
Mg^{2+}	Oxaloacetate (3)		0.39	11.8	10.6	11.6

[a] Oxaloacetate exists in equilibrium in aqueous solution: keto form (81%), enol (12%) and the gem-diol (7%) [14].
[b] 3,3-Difluoro-oxaloacetate exists predominantly in the gem-diol in aqueous solution [14].

nally proposed that two mechanistic scenarios were possible based on crystallographic and mutational studies. Furthermore, kinetic data has highlighted the efficacy of DMML in cleaving the C(2)-C(3) bonds of a variety of oxy-acids [6]. Substrates and inhibitors with a range of k_{cat} and K_m values have been reported and these offer great potential to test the validity of mechanistic proposals for this protein (Table 1). (2*R*, 3*S*)-dimethylmalate shows the largest k_{cat} at 19.2 s^{-1}, yet the replacement of the 3-methyl with an hydroxyl group to give oxaloacetate (**3**) leads to a 38 time fold drop in k_{cat}. Furthermore, it is reported that Mn^{2+} is catalytically more active than DMML containing the biologically more relevant Mg^{2+} ion by between 1.2–2.3 fold.

Recent computational studies in our group suggest that the catalytic mechanism involves 3 distinct steps, the first being the deprotonation of the substrate C(2) hydroxyl by Arg161 (Fig. 1) [22]. The role of Arg161 as the general base in the reaction was confirmed, and is consistent with similar observations in the mechanism of citrate synthase [23]. Proton transfer to Arg161 results in the C(2) oxy-nucleophile on the substrate. Next, the formation of a carbonyl group at the C(2) leads the breaking of the C(2)-C(3) bond. Proton transfer from Glu191 to the substrate C(4) carboxyl completes the formation of an aci-carboxylate intermediate and pyruvate. Step three involves the decomposition of the intermediate to propanoate through proton transfer from the Cys124 thiol to the C(3) atom, and the concomitant return of a proton to Glu191. It was found that the second step in the process was found to be rate determining for the natural substrate (**1**) [22].

In our efforts to better understand this class of protein, we investigate the catalytic mechanism of DMML further by studying a range of substrates and active site metals configurations, using molecular dynamics (MD) and a hybrid quantum mechanics/molecular mechanics (QM/MM) implementation. In a typical QM/MM study the protein macromolecule is treated using two different levels of theory [14,24–29]. The important active site residues that undergo chemical change, or directly influence the sequence of events in the catalytic reaction are treated using QM methods while the remainder of the protein is treated using more computationally efficient MM methods. Bonds that cross the QM-MM interface have their valence satisfied in the QM region using a hydrogen link atom.

The focus of this work is to ascertain whether the QM/MM model developed previously to study DMML [22] is capable of describing the subtle differences in the experimental k_{cat}s observed for a range of DMML substrates. Covalent inhibitors of ICL and MICL that exploit the catalytic cysteine residues have been reported on extensively [20,30]. A method that can correctly describe the chemical reactivity within the protein would therefore have value in the structure-based drug design of novel covalent inhibitors. Indeed, advantages of using covalent inhibitors to treat fungal species such as *Aspergillus niger* or *Escherichia Coli* are manifold; (a) covalent inhibitors are generally more potent than non-covalent types [31–33], (b) ICL-like targets are not present in humans meaning off-target effects are less likely [17] and (c) acidic antifungals will have intrinsically lower permeability and thus should show lower human absorption [34].

2. Computational methods

The 3D coordinates of DMML were taken from the RCSB protein data bank (PDB ID: 3FA3) [6]. The protein structure was prepared in the same way as that reported previously [22]. Missing amino acid atoms were added in Discovery Studio 4.0. The protonation states of amino acid were determined using PROPKA [35–37] at pH