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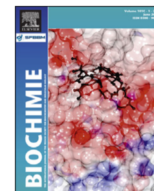

Research paper

Allantoinase and dihydroorotase binding and inhibition by flavonols and the substrates of cyclic amidohydrolases

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A B S T R A C T

Allantoinase and dihydroorotase are members of the cyclic amidohydrolases family. Allantoinase and dihydroorotase possess very similar binuclear metal centers in the active site and may use a similar mechanism for catalysis. However, whether the substrate specificities of allantoinase and dihydroorotase overlap and whether the substrates of other cyclic amidohydrolases inhibit allantoinase and dihydroorotase remain unknown. In this study, the binding and inhibition of allantoinase (*Salmonella enterica* serovar Typhimurium LT2) and dihydroorotase (*Klebsiella pneumoniae*) by flavonols and the substrates of other cyclic amidohydrolases were investigated. Hydantoin and phthalimide, substrates of hydantoinase and imidase, were not hydrolyzed by allantoinase and dihydroorotase. Hydantoin and dihydroorotate competitively inhibited allantoinase, whereas hydantoin and allantoin bind to dihydroorotase, but do not affect its activity. We further investigated the effects of the flavonols myricetin, quercetin, kaempferol, and galangin, on the inhibition of allantoinase and dihydroorotase. Allantoinase and dihydroorotase were both significantly inhibited by kaempferol, with IC_{50} values of 35 ± 3 μM and 31 ± 2 μM, respectively. Myricetin strongly inhibited dihydroorotase, with an IC_{50} of 40 ± 1 μM. The double reciprocal of the Lineweaver–Burk plot indicated that kaempferol was a competitive inhibitor for allantoinase but an uncompetitive inhibitor for dihydroorotase. A structural study using PatchDock showed that kaempferol was docked in the active site pocket of allantoinase but outside the active site pocket of dihydroorotase. These results constituted a first study that naturally occurring product flavonols inhibit the cyclic amidohydrolases, allantoinase, and dihydroorotase, even more than the substrate analogs (>3 orders of magnitude). Thus, flavonols may serve as drug leads for designing compounds that target several cyclic amidohydrolases.

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1. Introduction

The amidohydrolase superfamily comprises a remarkable set of enzymes that catalyze the hydrolysis of a wide range of substrates with amide or ester functional groups at their carbon and phosphorus centers [1–3]. Based on their functional and structural similarities to related enzymes, hydantoinase, allantoinase, dihydropyrimidinase, and dihydroorotase belong to the cyclic amidohydrolase family [4,5]. Hydantoinase is also known as dihydro pyrimidinase because of an overlap in substrate specificity [6]. Enzymes in this family [7–11], including imidase, are imide-hydrolyzing enzymes. Even though they are functionally similar, these enzymes have a relatively low amino acid sequence identity. These metal-dependent enzymes catalyze the ring-opening hydrolysis of the cyclic amide bond of each substrate in either five- or six-member rings in the metabolism of purines, pyrimidines, and many xenobiotics (Fig. 1A).

Allantoinase occurs in a wide variety of organisms, including bacteria, fungi, and plants, and a few animals. Allantoinase catalyzes the reversible hydrolysis of allantoin to allantoic acid, which is a key reaction in the biosynthesis and degradation of ureide required for the utilization of nitrogen in purine-derived compounds [12]. Allantoinase is a homotetrameric dinuclear metalloenzyme [13,14], but some allantoinases initially annotated as polysaccharide deacetylases [15] are metal independent. Thus, even without the allantoinase gene, some bacteria use allantoin to utilize nitrogen.

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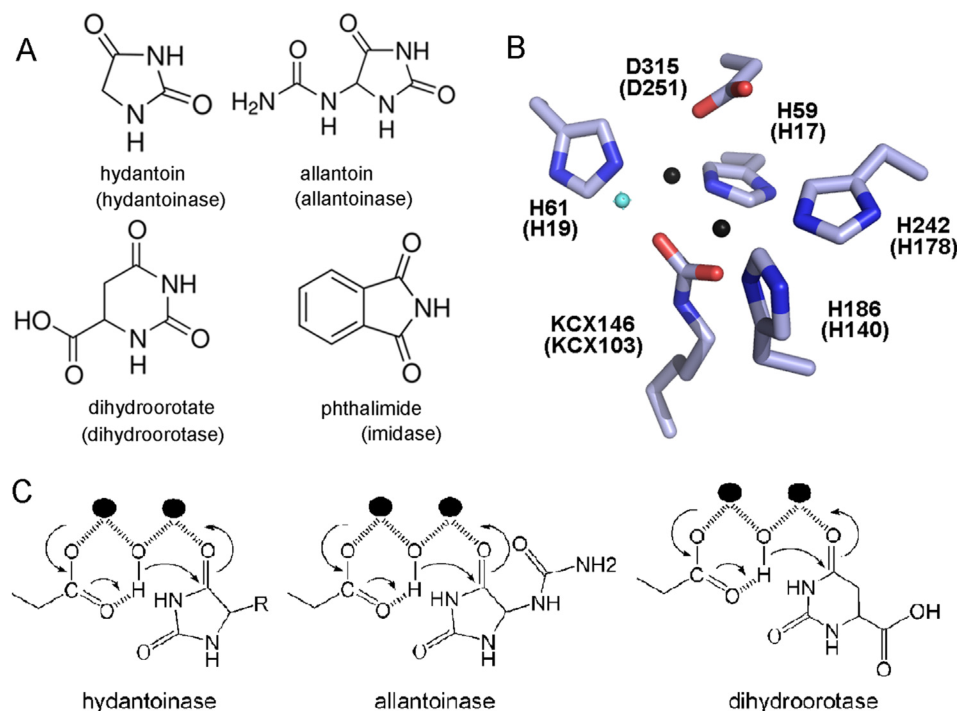

Fig. 1. Properties of the cyclic amidohydrolase family. (A) Substrate of hydantoinase/dihydropyrimidinase, allantoinase, dihydroorotase, and imidase. (B) The binuclear metal center within the active site of allantoinase and dihydroorotase (in parentheses). Allantoinase and dihydroorotase contains four histidine, one aspartate, and one post-carboxylated lysine residue, which are required for metal binding and catalytic activity, as revealed by their crystal structures. The coordinate was obtained from the Protein Data Bank (entry 3E74). KCX, a post-carboxylated lysine. The metal ions (in black) and a metal-bound water molecule (in light blue) are also shown. (C) The chemical mechanisms of hydantoinase, allantoinase and dihydroorotase. The hydrolysis of the substrates likely undergoes three steps: the hydrolytic water molecule must be activated for nucleophilic attack, and then the amide bond of the substrate must be made more electrophilic by polarization of the carbonyl-oxygen bond, and the leaving-group nitrogen must be protonated as the carbon–nitrogen bond is cleaved. The metal ions are shown as black circles.

Dihydroorotase catalyzes the reversible cyclization of carbamoyl aspartate into dihydroorotate in the third step of the de novo pyrimidine nucleotide biosynthetic pathway [16]. In mammals, this enzyme is part of the large multifunctional protein carbamoyl phosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD) [16–19]. However, in prokaryotic organisms, CADs are usually expressed separately and function independently [20] or form multifunctional complexes [17,21,22]. Similar to allantoinase, dihydroorotase is a metalloenzyme [21,23]. The active site of allantoinase [14] and dihydroorotase [23] contains four histidine, one aspartate, and one post-carboxylated lysine residue or a second aspartate residue, which are required for metal binding and catalytic activity, as revealed by their crystal structures (Fig. 1B) [13,24]. The presence of a post-carboxylated lysine in hydantoinase is also involved in binuclear metal center self-assembly [25] and increases the nucleophilicity of the hydroxide for catalysis [26]. Nevertheless, the substrate specificities of allantoinase, hydantoinase, and dihydroorotase may differ. However, whether the substrate of each enzyme competitively inhibits other enzymes in this family remains unclear.

Infections that are resistant to all antibacterial options have recently developed. Few therapies are effective against the six antibiotic-resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) [27]. Considering allantoinase and dihydroorotase are required for metabolizing purines and pyrimidines, blocking their activities would be detrimental to bacterial survival. In addition, allantoinase is not found in humans, and mammalian and prokaryotic dihydroorotases exhibit distinct differences. Thus, these enzymes may be promising therapeutic targets for developing antibiotics. Although some chelators inhibit allantoinase and dihydroorotase, they may be harmful to human health.

Flavonoids are the most common group of plant polyphenols and are responsible for much of the flavor and color of fruits and vegetables [28]. Over 5000 different flavonoids have been described, many which display structure-dependent biological and pharmacologic activities [29]. The six major subclasses of flavonoids are flavonols, flavones, flavanones, flavanols, anthocyanidins, and isoflavones [28]. Flavonols, which are composed of two aromatic rings linked by a heterocyclic pyran-4-one ring, are known to have antioxidant [30], antiradical [31], antiviral [32,33], and antibacterial activities [34,35]. In this study, we investigated the effects of the substrates of cyclic amidohydrolase and the flavonols myricetin, quercetin, kaempferol, and galangin on inhibiting the catalytic activity of allantoinase and dihydroorotase.

2. Materials and methods

2.1. Materials

All restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA), unless otherwise stated. All custom oligonucleotide primers were obtained from Invitrogen Corporation (Carlsbad, CA, USA). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Protein expression and purification

The construction of pET21e-*Kp*DHO expression vector for *Klebsiella pneumoniae* dihydroorotase expression has been previously

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