Substrate based inhibitors of Strawberry Dioxygenase: Homology Models

Alok Jha[*], Ramasare Prasad[**]

[*] Indian Institute of Technology Roorkee, India
[**] Indian Institute of Technology Roorkee, India

Abstract- The stereoselective oxidation of an unreactive alkane C-H bond is most often carried out by monoxygenases or oxygenases including dioxygenases which use iron as a cofactor and constitute a family of enzymes, e.g. Redox enzymes. A dioxygenase is an enzyme which incorporates both the atoms of molecular oxygen into kinds of substrates. The oxidative transformation reactions are common reactions during fruit ripening and Abscisic acid biosynthesis in strawberries. The main substrate of 2-OG (oxoglutarate) dependent dioxygenases is glutarate which is converted in succinate during flavor biogenesis. The dioxygenase currently studied belongs to 9-cis-epoxycarotenoid dioxygenases (NCEDs), which catalyzes the final step in Abscisic acid biosynthesis. Oxidative reactions catalyzed by 2-OG dependent dioxygenases are important mechanistic steps in the biosynthesis of variety of metabolites in plants as well as mammals, including flavor compounds and materials of medicinal and agrochemical importance, such as plant hormones e.g. gibberellins and abscisic acid, and antibiotics such as cepahlosporin and β-lactamase inhibitor clavulanic acid. Members of carotenoid cleavage dioxygenases (CCDs) family catalyze the oxidative cleavage of carotenoids at various chain positions leading to the formation of a wide range of apocarotenoid signaling molecule. Here, we report a selective inhibition of strawberry dioxygenase and assume that these inhibitor compounds can resolve functions of this diverse enzyme family. To explore the possibilities we have used a chemical approach to select designed inhibitors for different classes of carotenoid cleavage dioxygenases. We have used substrate based inhibitors such as oxoglutarate analogue Cyclohexanedione- Ca, Abamine derivative BAS8769 (N-[4-(4-Fluoro-phenyl)-thiazol-2-yl]-benzene-1, 4-diamine) and Dicamba which contains benzyl-aryl, cyclohexanone and benzoyl ring moieties respectively. We hypothesize that these are potent dioxygenase inhibitors and selectively inhibit dioxygenase enzymes that cleave carotenoids at 9,10,11,12 positions and also 2-oxoglutarate dependent dioxygenases e.g. 4-hydroxyphenylpyruvate dioxygenases (HPPDs).

Index Terms- dioxygenase, oxoglutarate, abamine, dicamba, HPPD, oxidative transformations

I. INTRODUCTION

The ripening of fruits involves a complex series of biochemical events which include tissue changes in texture, aroma, coloration, flavor and firmness. On the basis of fruit differential respiration and ethylene effects, climacteric and non-climacteric fruits have been classically defined and the molecular mechanism of their ripening have been the focus of study over the past decades. The molecular mechanism of ripening of climacteric fruit have been described as ethylene perception and signaling transduction. A model for non-climacteric fruit ripening has been suggested for strawberry which involves Abscisic acid (ABA) perception and signaling transduction. The plant hormone Abscisic acid (ABA) is a key hormone involved in a broad spectrum of growth and development processes including seed maturation and dormancy [(3,4)], root and shoot growth [(5)], drought responses [(6,7)] and nutrient depletion[(8)]. In maize, the world's most productive cereal crop, a 9-cis-epoxycarotenoid dioxygenase (NCED) catalyzes the rate-limiting step in ABA biosynthesis [(9,10)] -the oxidative cleavage of the 11,12 carbon-carbon double bond of 9-cis-epoxycarotenoids, either 9-cis-violaxanthin or 9-cis-neoxanthin [(11)]. The C15 aldehyde, xanthoxin, is oxidized and converted through two subsequent reaction to the biologically active ABA [(12,13)]. Thus, NCEDs including VP14 are key regulators that determine ABA levels [(13)], which in turn control ABA-regulated processes. Dioxygenases have been classified on the basis of substrates; the carotenoid cleavage dioxygenases (CCDs) catalyze cellular processes by carotenoid cleavage. The carotenoid cleavage dioxygenases include 9-cis-epoxycarotenoid dioxygenase (NCED) which regulates the rate-limiting step in ABA biosynthesis by cleaving the carbon-carbon double bond of 9-cisepoxycarotenoid. Other group of dioxygenases include 2-oxoglutarate (2-OG) or α-ketoglutarate dependent dioxygenase such as prolyl hydroxylase, catechol dioxygenase, tryptophan-2,3-dioxygenase and indoleamine-2,3-dioxygenase, 4-hydroxyphenylpyruvate dioxygenase etc. Prolyl hydroxylase regulates hypoxia inducible factor 1 α (HIF 1α), in which proline residues are hydroxylated [(14)]. Both tryptophan-2,3-dioxygenase and indoleamine-2,3-dioxygenase participate in tryptophan metabolism via Kinurenine pathway[(15)]. The hydroxyphenylpyruvate dioxygenases (HPPDs) are different from carotenoid cleavage dioxygenases (CCDs) as they participate in succinate biosynthesis by converting 4-hydroxyphenylpyruvate in to 2, 5-dihydroxyphenylacetate (homogentisate) with the concomitant release of CO_2 [(16,17)]. This transformation involves decarboxylation, aromatic hydroxylation and substituent migration in a single catalytic cycle. The reaction mechanism is similar to those catalyzed by the α-ketoacid dependent superfamily of oxygenase enzymes [(18)]. These ketogenic and glucogenic products have a direct energetic contribution; in higher organisms the pathway serves additional functions.

Carotenoids are synthesized in plants and microorganisms as photoprotective molecules and important components in animal diets, an example being β-carotene (pro-vitamin A). The oxidative cleavage of carotenoids occurs in plants, microorganisms and animals and leads to the release of a range of apocarotenoids that function as signaling molecules with diverse functions [19]. In insects the visual pigment retinal is formed by oxidative cleavage of β-carotene β-15,15′-dioxygenase [20]. Retinal is produced by an orthologous enzyme in vertebrates, where, it is converted to retinoic acid, which is also a regulator of differentiation during embryogenesis [21]. A distinct mammalian CCD is believed to cleave carotenoids asymmetrically at the 9,10 position [22], that is involved in the metabolism of dietary lycopene [23]. The plant volatiles β-ionone and geranylacetone are produced from an enzyme that cleaves at the 9,10-position [24] and the pigment α-crosin found in the spice saffron results from an 7,8-cleavage enzyme [25].

The advent of detailed structural information on the molecular target sites of agrochemical now allows a rational target site based approaches to improve both selectivity and potency towards the pathogen species and away from nontarget organisms. One enzyme that is potentially complaisant with such an approach is 4-hydroxyphenylpyruvate dioxygenase (HPPD), the target site for recently commercialized herbicides and therefore of great interest for the design of novel herbicides [26, 27]. In mammals the enzyme has an important role in the catabolism of tyrosine. Deficiency of these enzymes in humans causes type III tyrosinemia, a rare autosomal recessive disorder characterized by elevated serum tyrosine levels, neurological symptoms and mental retardation [28-31]. Inhibitors of HPPDs have found use as drugs for the treatment of type I tyrosinemia by blocking the formation of toxic catabolites derived from tyrosine in these disease conditions [32]. In plants, HGA formed by HPPD activity is utilized as the aromatic precursor for tocopherols and plastoquinone [33]. Plastoquinone is the redox cofactor for phytoene desaturase, a key enzyme in the biosynthesis of photoprotectant carotenoids [33]. The loss of these essential phytoprotectants result in the intense and characteristic bleaching of new plant growth by application of HPPD inhibitors herbicides leading to plant death.

II. Substrate based Inhibitor design

Considerable success has been obtained in probing function and substrate specificity of CCDs in their native biological contexts, particularly in plant species with simple genetic systems or that are amenable for genetic approaches. CCDs are often active against a broad range of substrates and in many cases the true in vivo substrate of a particular CCD remains unknown. Therefore, to investigate both apocarotenoid and CCD functions in their native cellular environment different small molecules can be applied easily to a broad range of species, their application can be controlled to provide detailed studies of biological functions, and individual proteins or whole protein classes may be targeted by varying the specificity of the small molecules. Notably, functions of plant hormones gibberellins, brassinosteroids and abscisic acid have been successfully probed using this approach by adapting triazoles to inhibit specific cytochrome P450 monoxygenases involved in the metabolism of these hormones [34]. In the case of CCD family the tertiary amine Abamine [35] and the more active AbamineSG [36] have been reported as specific inhibitors of NCED, while Abamine was used to show new functions of abscisic acid in legume modulation [37]. However, other selective inhibitors for other types of CCD are also known, CCD inhibitor based on hydroxamic acid where variable chain length was used to direct inhibition of CCD enzymes that cleave carotenoid at specific positions. We, therefore, report a novel combination of inhibitors for dioxygenase enzyme superfamily and demonstrate the use of such novel strawberry dioxygenase inhibitors to control disadvantages of the dioxygenase in the model plant.

III. Results and Discussion

Dioxygenase selective inhibition:

9-cis-epoxycarotenoid dioxygenases are proposed to be a dioxygenase with a mechanism involving a carbon-carbon intermediate followed by formation of a dioxetane ring or Criegee rearrangement prior to cleavage[11,38,39]**.** The dioxygenase inhibitors which have been previously reported elsewhere for different types of dioxygenases are most likely based on chemical structure modifications in various dioxygenase substrates or natural inhibitors. It was reported that the tertiary amine Abamine is a reversible competitive inhibitor of NCEDs and it inhibited Abscisic acid biosynthesis in plants [35] and Abamine SG with an extended three carbon linker between the methyl ester and nitrogen was subsequently developed with an improved activity [36]. Similarly, the 2-oxoglutarate (2-OG) dependent dioxygenase substrate 2-oxoglutarate analog Cyclohexanedione-Ca may inhibit this type of dioxygenases. Chemical compounds containing amines, imidazole are potential dioxygenase inhibitors such as plant hormone auxin based synthetic compounds 2,4-D (2,4-Dichloroacetic acid) and Dicamba (2-methoxy 3,6 chloroacetic acid) can inhibit dioxygenase functions. These chemical compounds act on the methyl group of the substrate. Here, we report the 2-OG glutarate analog Cyclohexanedione-calcium or Cyclohexanedione-Ca, Abamine derivative BAS8769 and Dicamba as potential 2-OG dependent dioxygenase, 9-cis-epoxycarotenoid dioxygenase and 4-hydroxyphenylpyruvate dioxygenase inhibitors. The precise mechanism of action of Abamine is uncertain but our hypothesis was that the protonated amine mimics a carbocation intermediate in the catalytic mechanism with the oxygenated aromatic ring bound in place of the hydroxyl-cyclohexyl terminus of the carotenoid substrate [38]. Inhibition may be due in part by chelation of the essential metal ion cofactor by the methyl ester of Abamine. However, a lot of Abamine derivatives have been reported, for example an Abamine derivative containing an acid group (-COOH) in place of the methyl ester was not active [36] even though theoretically this should be more effective at binding the iron cofactor. Hydroxamic acids are known to act as inhibitors of several classes of metalloenzymes such as matrix metalloprotease by chelation of the essential metal ion cofactor [40]. Therefore, we selected hydroxamic acid derivative or Abamine derivative N-[4-(4-Fluoro-phenyl)-thiazol-2-yl]-benzene-1,4-diamine or BAS8769 in which hydroxyl-cyclohexyl terminus of the carotenoid has been mimicked as above by an oxygenated ring and the hydroxamic functional groups are

positioned at variable distance from the aromatic ring. We searched for different aryl-C_3N analogues, aryl-C_2N and aryl-C_1N analogue and selected BAS8769 from the database. The compound BAS8769 contains 4-fluorobenzyl group that has been found to promote activity in the Abamine series [35], also the thiazole group includes in the selection of hydroxamic acid or Abamine derivative BAS8769. The coupling of the appropriate acid with a substituted o-benzyl hydroxylamine and the carbon spacer from the cyclohexyl moiety was involved in the selection of BAS8769. Our hypothesis was that the halogenated i.e. chlorinated or fluorinated aromatic ring side chain compounds can be potential inhibitors for this class of dioxygenases, therefore, the study remains focused on inhibitors containing such aromatic rings e.g. 4-Fluoro-phenyl rings in BAS8769, chloroacetic acid in Dicamba. The 2-OG dependent dioxygenase inhibitor Cyclohexanedione-Ca, which is 2-OG analogue, contains calcium as calcium plays important role in the diverse functions of dioxygenases. Biochemical studies of plant species susceptible to Cyclohexanedione herbicides indicated that these herbicide inhibitors inhibit acetyl co-A carboxylase in grasses [41], however, these inhibitors could be effective also in broadleaf plants depending on the sensitivity.

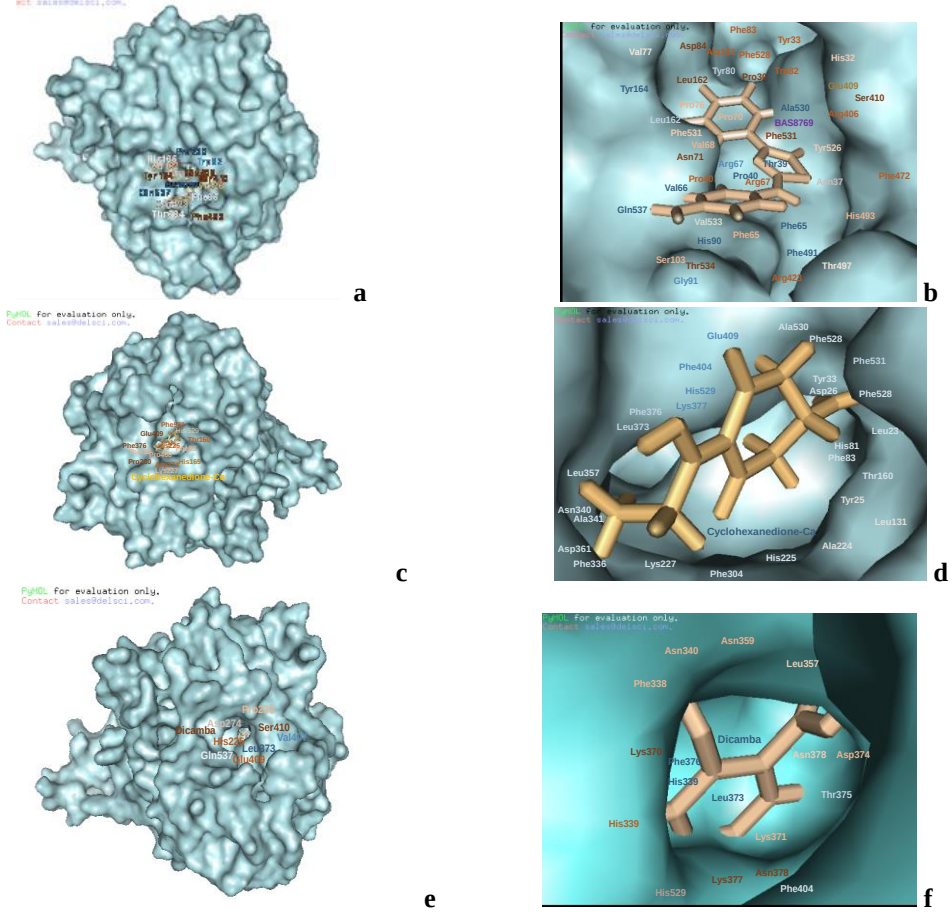
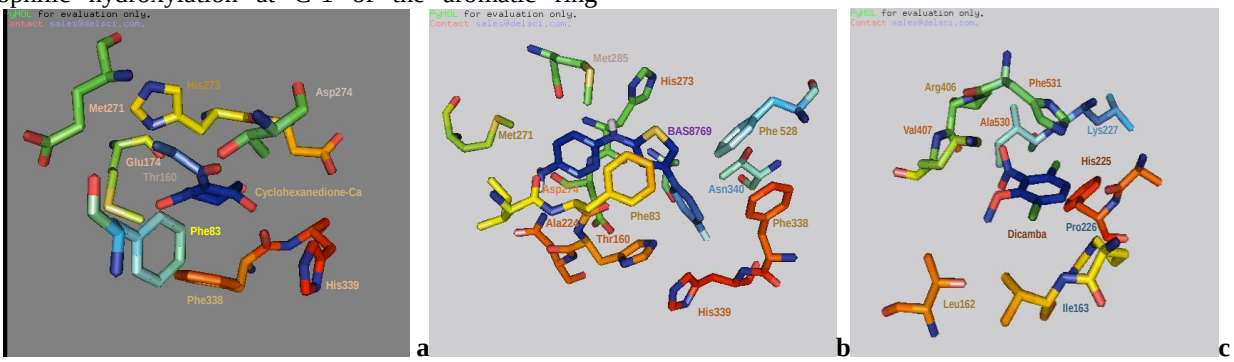


Fig. 1 Dioxygenase substrate based inhibitors: a,b represent abamine derivative compound BAS8769. **c,d** represent oxoglutarate analogue Cyclohexanedione-ca. **e,f** represent Dicamba. The correct positioning of hydroxamic ring in the active site is required for competitive binding, the phenyl or benzoyl rings of inhibitors are anchored in the hydrophobic base of the substrate binding pocket. Cyclohexanedione-Ca is in a hanging position supported by Phe528 residue, while Phe531 and Arg67 form the base of substrate binding pocket. BAS8769 and Cyclohexanedione-Ca shows superficial interaction with the residues in the substrate binding pocket whereas Dicamba is deeply anchored in the substrate binding pocket and its benzoyl moiety and chloroacetic side chain are interacting with residues His225 and Phe531 respectively, that are involved in forming walls of substrate binding pocket.

The interaction of BAS8769 and Cyclohexanedione-Ca and Dicamba with dioxygenase involves certain conserved residues. However, residues interacting with BAS8769 and Cyclohexanedione-Ca are similar but Dicamba has different interaction pattern, this indicates similarity and difference in the chemical structure of these substrates. All these substrates are aromatic ring compounds with varying side chains, BAS8769 and Cyclohexanedione-Ca are modified as the aromatic ring contains Fluoro-phenyl and Thiazole side chains in BAS8769 and in Cyclohexanedione-Ca the aromatic ring contains C_2H_5O- and $CH_3(CH_2)_2$- and C_2H_5S- groups as side chain. The synthetic auxin inhibitor Dicamba contains dichloro- and methoxy- groups on benzene ring, which makes electron centered at the aromatic ring and a potential target for electrophilic reactivity, while in some cases nucleophilic reactions are also a possibility. We propose a reaction mechanism for binding of these substrates, the

way of access to the catalytic site and the mode of binding in the substrate binding pocket is crucial to determine accurately how the inhibition occurs, however, the chemical groups on inhibitors and interacting residues can indicate the mechanism behind the inhibition process. The co-ordination geometry of inhibitor binding is consistent with that of other enzymes of dioxygenase superfamily thus we are able to propose that certain specific mechanism is applied in such cases. The most plausible explanations to the mechanistic possibilities are the presence of specific motifs which lead to activation of the substrate and reactions associated with enzymatic activity. The proposed mechanism involves the ionic or radical mechanism for substrate intermediate generation which in turn inhibits the dioxygenase enzyme activity. The reaction of dioxygenase substrate with dioxygen to form a hydroperoxide which upon a nucleophilic attack on neighboring alkene forms a dioxetane which in the presence of water gets exposed to form epoxide. The iron-oxo intermediate, then carries out hydroxylation at benzylic position or electrophilic hydroxylation at C-1 of the aromatic ring followed by a 1, 2-alkyl shift e.g. Dicamba [1,2]. The inhibition is possible and evidences suggest that the substrate analogues in which hydroxymethyl substituent is positioned in an axial orientation with the cyclohexanone ring can be potential dioxygenase inhibitors, indicating that the conformations adopted by the hydroperoxide is important e.g. Cyclohexanedione-Ca. The exposure of mononuclear iron (II) complex to oxygen leads to the concomitant oxidative decarboxylation of a benzyolformate ligand and the hydroxylation of a nearby aryl ring in the ligand e.g. BAS8769. The reactivity of this complex therefore effectively mimics the reactivity of this class of dioxygenases. The type of hydroxylation reaction in which the reaction proceeds via three oxygens, two oxygens are derived from dioxygen and another one is derived from water, this labeling pattern can be explained by a similar transformation to give an epoxyquinone intermediate, followed by base- catalyzed epoxide opening by water and ketone reduction [1,2].



a b c

Fig. 2 Dioxygenase inhibitor interactions. a. Cyclohexanedione-Ca b. BAS-8769 c. Dicamba

The residue Phe83 interacts with the oxygen atom of the cyclohexanone ring of Cyclohexanedione-Ca. Other residue Thr160 forms polar interaction with the cyclohexanone ring, whereas, in BAS8769 these residues interact differently. Phe83 interacts with the benzyl-aryl ring of BAS8769 by forming a π-π interaction, while Thr160 is interacting with the carbon atom of the benzyl-aryl ring. Other residues, Glu174 including active site residues His339 and His273 in Cyclohexanedione-Ca, are interacting with the carbon atoms of cyclohexanone ring. These are hydrophobic interaction but in the case of BAS 8769, His273 forms polar interaction with the hydrogen atom, while its carbon atom interacts with nitrogen atom of cyanoaryl ring of the substrate. His273 also forms π-π interaction with the benzyl ring carbon atoms and with hydrogen atoms of the phenyl ring of BAS8769; it forms cation-π interaction. The residue Phe338 is interacting with a hydrophobic interaction with carbon atoms of cyclohexanone ring and with oxygen atom it forms a hydrogen bond or a weakly polar interaction in Cyclohexanedione-Ca. In BAS8769, Phe338 forms a π-π interaction with the carbon atom of benzyl-aryl ring. The residue Phe338 also interacts with the sulfur atom S1 of thiazole side chain of BAS8769, which suggests that Phe338 is responsible for dioxygenase inhibition by BAS8769. The sulfoxide elimination is an important tool to double bond generation in aromatic rings, the Phe338 interaction with sulfur atom S1 of thiazole group can generate double bond in the phenylalanine ring and the discriminate binding may participate in dioxygenase inhibition and can also be associated with increased glutathione-S-transferase activity enhancing glutathione conjugation as observed in triazine herbicide inhibitors. This is a common mechanism of atrazine detoxification in some grasses and broadleaf weeds. [42-45] Moreover, Phe338 can also induce substrate selectivity by its characteristic cis-cis conformation [46]. The residue Phe528 is interacting with benzyl-aryl ring carbon atoms by forming π-π interactions, while its carbon atom forms a halogen bond or interact with the fluorine atom F1 of fluoro-phenyl ring of BAS8769. It suggests that Phe528 along with forming the base of the substrate binding pocket, participate to position the hydroxamic acid cyclic end group in the active site to facilitate the inhibitory activity of BAS8769 in this class of dioxygenases.

Synthetic auxins act as mimics of natural auxin and are categorized into different classes based on the position of their carboxycyclic acid moieties on their aromatic rings. The classes include phenoxyalkanoic acids (e.g.2,4-D), benzoic acids (e.g. Dicamba) and pyridine-carboxylic acids (e.g. picloram) [51]. Overall, effects of auxinic herbicides can be divided into three consecutive phases in the plant: stimulation of abnormal growth and gene expression, inhibition of growth and physiological responses and senescence and cell death [52]. The Dicamba interaction with specific amino acid residues in dioxygenase can serve the basis for its mode of actions. The residue His225 is interacting with the oxygen atom of benzoic ring by a polar

interaction or a hydrogen bond, while, forms π-π interaction with carbon atoms of the benzoyl moiety, which suggests that His225 can be participating in forming a catalytic triad of homologous residues mechanistically to facilitate the inhibitory activity of Dicamba in this class of dioxygenases. His225 also interacts with the chlorine atom of chloroacetic acid group of Dicamba. The chlorine atom of Dicamba also interacts with the residue Phe531. Other residues, Leu162 and Ala530 can also interact with the chlorine atom. The interactions of Pro226 with the oxygen atom of benzoyl moiety and the nearby residue, Lys227, which provides some stability to the structure and its ammonium group, can be neutralized by the oxygen atom of benzoyl moiety, which involves carbon atoms of Lys227. Other residue, Arg406 interacts with weak polar or hydrophobic interactions with carbon atoms of the benzoyl ring and also with chlorine atom of the chloroacetic acid side chain. Val407 can also interact with the chlorine atom of the chloroacetic acid side chain, while, Thr412 interacts with the benzoyl ring carbon atoms by hydrophobic or weak polar interactions. We suggest that these interactions could have functional significance for Dicamba mode of actions. The purpose of selecting inhibitors of these classes for strawberry dioxygenase was that the inhibitors based on structural mimic of the substrate position an iron chelating hydroxamic acid group within the active site. The positioning of hydroxamic acid group within the active site depends on the distance between the hydroxamic acid group and an aromatic ring, all the inhibitors that we have selected can achieve this, as the distances match within the carotenoid substrate between the proximal cyclic end-group and the cleavage site. Dioxygenase structures of known types indicate that cleavage position is determined by the distance between the Fe(II) catalytic centre and the opening of the long non-polar tunnel that allows the access to carotenoid substrates [47]. The cleavage of monocyclic γ-carotene in Nostoc sp. CCD(NosCCD), for example occurs at 7′,8′-position where the proximal terminus is linear, but at 9,10-position where the proximal terminus has a more compact cyclic end group [48], indeed, this suggested that the cyclic end group arrested at the entrance of the tunnel. We predict from the structure model of strawberry dioxygenase for inhibitor mechanism, that the aryl-C_1N,aryl-C_2N, and aryl-C_3N compounds would be selective for 7,8, 9,10 and 11,12 cleavage reactions [49,50], however, we also speculate that these inhibitors may have 15-15′ specificities [49], they all still maintain a somewhat greater selectivity toward the 9,10 and 11,12 cleavage. These inhibitors Cyclohexanedione-CA, BAS8769 and Dicamba may exhibit different activity pattern with 9, 10 and 11, 12 cleavage enzyme activity with significant inhibitory activity in both plant and human dioxygenases. This also indicates that the variant of hydroxamic acid inhibitors are able to distinguish between enzymes that have similar activities but highly divergent primary structure e.g. Tryptophan-2, 3-dioxygenase, Indoleamine-2, 3-dioxygenase in humans.

IV. Conclusions

The mode of activation and paucity of pharmacological tools have made dioxygenases one of more challenging oxygenases to characterize and an important target for the development of herbicide inhibitors and also useful drugs. The structure model offers insights into the high affinity binding of structural mimics of various substrates. Therefore, this structure and inhibitor complex will provide a template for development of strawberry dioxygenase inhibitors and the development of inhibitors for other dioxygenases to probe their biological roles. Further efforts will focus on improving the possibility of inhibitor compounds bound to an active state dioxygenase structure.

V. Methods Summary

In the homology modeling protein sequence should be analyzed for certain specificities before generating structure model to validate the structure. Therefore, we retrieved the strawberry dioxygenase amino acid sequence from NCBI database (www.ncbi.gov.in), and further analyzed it for sequence based similarity and multiple alignments against other protein sequences. With more than 30% similarity and less gap in the alignment, the template 3NPE_A served as an ideal template for structure model generation. The structure model was generated from geno3D server (**geno3d**-pbil.ibcp.fr/). The dioxygenase sequence was analyzed for secondary structure and topology predictions, using PSI-PRED (128.16.10.201/**psipred**/) and EMBL-EBI (www.**ebi**.ac.uk/) tool ProFunc for protein functions. The multipass model expectation value threshold 0.002 in the expectation value of 10 and the alignment was done in the matrix BLOSUM62 for model generation. We deduced substrate 3D structure from PubChem compound. (www.ncbi.nlm.nih.gov). Dicamba=CID3030, Cyclohexanedione-Ca=CID13006, N-[4-(4-Fluoro-phenyl)-thiazol-2-yl]-benzene-1,4 diamine=BAS08769360. The substrate docking studies were performed by using Hex (**hex**.loria.fr/), and dockingserver, (www.**dockingserver**.com/). The pictures were generated by PDB viewer PyMol (www.**pymol**.org/).

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AUTHORS

First Author – Alok Jha, Research Scholar, Department of Biotechnology, Indian Institute of Technology, Roorkee, Roorkee. E-mail: alok.gene@gmail.com, Phone: +919897594489

Second Author –Prof. Dr. Ramasare Prasad, Professor and Head of the Department, Department of Biotechnology, Indian Institute of Technology, Roorkee, Roorkee.
E-mail: rapdyfbs@iitr.ernet.in, Phone: 91-01332-285791

Correspondence Author –
Prof. Dr. Ramasare Prasad, Professor and Head of the Department, Department of Biotechnology, Indian Institute of Technology, Roorkee, Roorkee. E-mail: rapdyfbs@iitr.ernet.in, Phone:91-01332-285791