RESEARCH PAPER | Biotechnology | Volume : 6 | Issue : 2 | FEBRUARY 2016 | ISSN - 2249-555X

Cholesterol binding to the carotenoid binding site in Strawberry Dioxygenase

KEYWORDS: Dioxygenase, 9-cis-epoxycarotenoid, α-epoxycholesterol, lipoprotein, Strawberry

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ABSTRACT *The 2-oxoglutarate (2-OG) dependent dioxygenases have an absolute requirement for Fe (II) and catalyze a variety of two electron oxidations, including hydroxylation, desaturation and oxidative ring closure reactions. Here, we report a novel α-epoxycholesterol binding to the 9-cis-epoxycarotenoid binding site of dioxygenase in strawberry. The structure model suggests an unusual mode of α-epoxycholesterol binding in the 9-cis-epoxycarotenoid binding pocket that explains the possible location and mechanism for α-epoxycholesterol or lipid oxidation while transferring oxidized lipid in the low density lipoprotein (LDL) and high density lipoprotein (HDL) from chylomicron and chylomicron remnants (CM/CR). The structure can sufficiently produce a measureable improvement by supporting theories pertaining to the oxidation of lipids by dioxygenase. We anticipate that the dioxygenase-9-cis-epoxycarotenoid and α-epoxycholesterol structure complex model will aid to the development of cholesterol lowering drugs and in understanding the mechanism of dietary lipid oxidation.*

Introduction:

Strawberry has potential health benefits, as it contains high fiber content, low fats, high anti-oxidants and flavonoids, which help reducing cholesterol levels in the blood. Strawberry fruit extract has properties of treating diabetes, obesity, cardiovascular and other heart related diseases. The extracts from strawberries activate a protein in our bodies called, Nrf2, which is shown to increase antioxidants and other protective activities. This protein works to decrease lipids and cholesterol, the very thing which can lead to cardiovascular problems.[19] Previously, eating strawberries have been found to counter post meal-blood glucose and low density lipoprotein or 'bad' cholesterol, and therefore, decrease risk of diabetes and heart disease but strawberry extracts have been proved to be actively stimulating proteins, which protect us against diseases. The science behind how, strawberries especially, dioxygenases may work to increase our in-built-defenses to keep cells, organs and blood vessels healthy and that can reduce the risk of developing cardiovascular problems, such as heart disease and diabetes. These results indicate that strawberry extracts, specifically leaf extract is a direct, endothelial-dependent vasodilator, action of which is mediated by Nitric oxide (NO) and cyclooxygenases and dioxygenases. [1] Beneficial effects of strawberries also include anti-thrombotic effects, which are mostly attributed to the phenolic compounds that are found in large quantities in strawberry fruits. The most commonly studied phytochemicals have been extensively phenolic compounds in plant foods as their intake has been associated with reduced susceptibility of LDL to oxidation, reduced platelet aggregation, increased production of nitric oxide and increased human plasma anti-oxidant capacity.[2] Thus, strawberry fruits and leaves would be a good experimental material for studying physiology of polyphenolic compounds, as well as flavonoids and procyanidins in humans. We want to add strawberries, as a source of antioxidants and its dietary portfolio as a cholesterol-lowering-diet and to improve its anti-oxidant effects. The strawberry supplementation results in the greater reduction in oxidative damage to low density lipoprotein (LDL), measured as thiobarbituric acid reactive substances in the LDL maintaining low blood lipid and diet palatability.[3] The oxidized LDL enhances accumulation of monocyte in the subintimal spaces and macrophages conversions with increased uptake of LDL by scavenger receptors. The subintimal lipid accumulations and cell death have been considered to be a part of the process of atherogenesis.[3,14-16] Consequently, in the long term, lower levels of circulating oxidized LDL after the consumption of strawberries may reduce the risk of cardiovascular diseases. Increase in the uptake of fruits and vegetables are associated with reduction in diseases, such as chronic heart disease ascribed in part to their soluble (viscous) fibre content, which has been noted to reduce serum cholesterol and also in part to their source of potassium, which has been suggested to reduce blood pressure. [3] Since, strawberries do not show much weight gain, so, by dietary approach strawberries can be made a substitute for "luxury" foods including calorie rich desserts. There are several prescribed medications for lowering cholesterol and reducing the risk of cardiovascular diseases. Statins are some cholesterol lowering drugs but their long term use is linked to many side effects related to pancreas, liver, muscles, kidney etc. are very common. It is also suggested that use of lipid lowering therapies may increase the risk of pancreatitis, some pharmacoepidemiology studies have claimed that statins may cause pancreatitis, although lipid guidelines recommend fibrate therapy to reduce pancreatitis risk in persons with low hypertriglyceridemia.[4] So, using fibrates or statin drugs for lipid lowering therapies could raise questions regarding the choice of lipid-modifying agents in patients with hypertriglyceridemia. However, statins are better supported by the available data than fibrates for preventing pancreatitis and life modifications remain also important to improve lipid profiles in the individuals with hypertriglyceridemia.

Methods and Results:

A protein sequence should be analyzed for certain specificities before generating structure model to validate the concept of homology modeling. Therefore, we deduced the strawberry dioxygenase amino acid sequence from NCBI database (www.ncbi.gov.in), and further analyzed it for sequence based similarity and multiple align-

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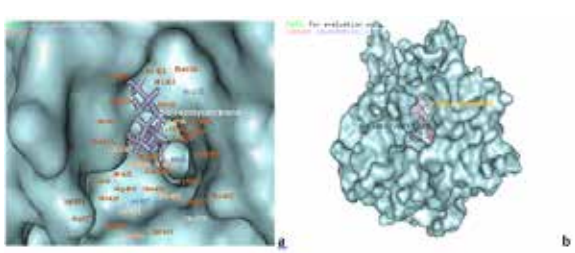
ment against other protein sequences. With 42.14% similarity and less gap in the alignment, the template 3NPE_A served as an ideal template for structure model generation. The models were generated with the multipass model expectation value threshold 0.002 in the expectation value of 10 and the alignment was done in the matrix BLOSUM62. We deduced substrate 3D structure from PubChem compound. The substrate docking studies were performed by using Hex and dockingserver. The pictures were generated by PDB viewer PyMol.

Dioxygenase substrates:

The overall structure of dioxygenase is an outward facing conformation and the substrates are likely to be trapped within the centre of the trans-membrane domain completely or minimally occluded from the intracellular side yet solvent accessible through the extracellular side through a channel or pore which is of 8-40A° diameter and presumably not too wide for the substrate to escape. The structure model of dioxygenase with and without substrate may represent an important expansion to the collection of dioxygenase conformations. In agreement to other dioxygenase structures of known types, strawberry dioxygenase structure contains a β-propeller containing 8 antiparallel β-sheets and 8 α-helices, while two of them are interacting with the membrane surface on top of the β-propeller at N- and C-terminals. In the intracellular side mainly branched chain residues are interacting with the cytosolic trans-membrane helical domain by strong and weak polar interactions without forming loops. The amino acid residues that constitute the intracellular helical domain are highly conserved in dioxygenases. Particularly, most of the residues that mediate inter-domain interactions are largely invariant in strawberry dioxygenase and maize VP14.[13] On this basis, we assume that similar helical domain interacting with membrane surface and cytoplasmic domains might exist in other types of dioxygenases and may be interacting with trans-membrane portion of the dioxygenase as observed in strawberry dioxygenase structure.

β-strands with medium size loops are discontinuous which can facilitate conformational changes during substrate transport as dioxygenases belong to the group of enzymes where substrate binding is a dynamic process which means that the substrates are channelized after binding to the dioxygenase enzyme for further processes. Although, dioxygenases have variety of substrates, which can bind to the active site but as the main function of dioxygenases in strawberry seems to be oxidative transformations and in the carotenoid and Abscisic acid biosynthesis by cleaving carbon-carbon double bonds of 9-cis-violaxanthin or 9-cis-neoxanthin in the rate limiting step, the maximal approach has been to investigate the 9-cis-epoxycarotenoid as a substrate considering strawberry dioxygenase as a 9-cis-epoxycarotenoid cleaving enzyme (NCED). [17-18]



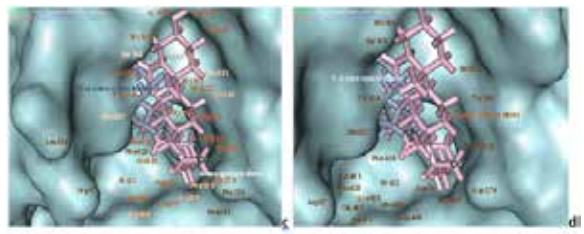


Figure. 1. α-epoxycholesterol binding to the 9-cis-epoxycarotenoid binding site. (a, b, c, d)

A Novel α-epoxycholesterol binding site in Dioxygenase: 9-cis-epoxycarotenoid binding site:

The dioxygenases structure in consistent with other dioxygenase structures contains a tunnel running throughout the structure and the active site is located at the central axis of the tunnel pivoted by four Histidine residues His225, His273, His339 and His529, that are bound to the Fe^{2+} metal ion octahedrally. As in dioxygenases, substrate binding is a dynamic process, any substrate has to reach to the catalytic site and should dissociate after the activation reaction. The dioxygenase structure, however, is compatible to indicate the substrate access but the mechanism of access requires further detailed investigations. The dioxygenase substrate 9-cis-epoxycarotenoid binds to an unusual location in the dioxygenase structure that is very close to the extracellular surface while some of the substrates bind much deeper in the substrate binding pocket. The 9-cis-epoxycarotenoid binding pocket is composed of different β-sheets, α-helices and loops that form a cage like strcuture which surrounds the substrate (Fig.1.). Residues such as Lys370, Lys372, Lys377, Asn340, Asn378 and His529 contribute to the formation of open end of the 9-cis-epoxycarotenoid binding pocket, whereas, residues Arg67, Gly467, Ile471 are forming the closed end of the binding pocket. The α-helical domain and loops between β-strands containing residues Arg406, Val407, Glu409, Pro405, Leu424 and Ser426 form the base of the 9-cis-epoxycarotenoid binding pocket. The residues involved in the formation of 9-cis-epoxycarotenoid binding pocket are polar rigid and rather hydrophobic providing support to anchor the substrate in the substrate binding pocket. The dioxygenase coordination that is common in the class of enzymes such as photosystem II and Fumerate reductase [7,8], suggested that the dioxygenase is greatly involved in fatty acid and amino acid metabolism, therefore, we speculated that lipids and cholesterol may also be ideal substrates for strawberry dioxygenases. Here, we report a novel α-epoxycholesterol binding to the 9-cis-epoxycarotenoid binding site, which has potential strctural and functional implications.

Alpha-epoxycholesterol binding:

The etiology of atherosclerosis is complex and multifactorial[15] but there is extensive evidence indicating that oxidized lipoproteins may play a key role. At present, the site and mechanism by which lipoproteins are oxidized are not resolved, and it is not clear if oxidized lipoproteins form locally in the artery wall and/or are sequestered in atherosclerotic lesions following the uptake of circulating oxidized lipoproteins. We have been focusing our studies on demonstrating structurally, that such potentially atherogenic oxidized lipoproteins in the circulation are at least partially derived from oxidized lipids in the diet.[15] Thus, the purpose of our work has been to determine in humans whether oxidized dietary oxidized fats such as oxidized fatty acids and oxidized cholesterol are absorbed and con-

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tribute to the pool of oxidized lipids in circulating lipoproteins.

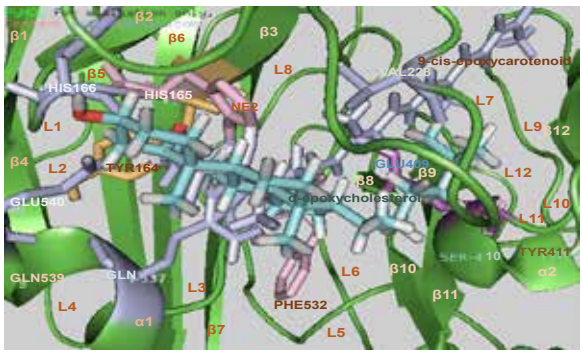

Figure. 2. α-epoxycholesterol and 9-cis-epoxycarotenoid binding site

Residues such as Glu409, Phe532 and His529 are interacting with the 9-cis-epoxycarotenoid by forming hydrogen bonds. Glu409$^{(-0.17)}$ and His529$^{(-15.67)}$ are involved in polar interactions with oxygen atoms of 9-cis-epoxycarotenoid by their oxygen and nitrogen atoms respectively with corresponding decomposed interaction energies of -0.17 and -15.67 kcal/mol (Fig.2). Residues that interact with carbon atoms of 9-cis-epoxycarotenoid include His225$^{(29.15)}$ which is located on a loop between α-helix α_5 (Tyr197-Lys199) and β-strand 9(Met235-Thr237) (β-sheet D) containing β-turn 25 (Thr223-Pro226) and γ-turn5 γ5(Thr223-His225). The carbon atoms of His225 $^{(29.15)}$ form a cation-π interaction with the H1 atom of 9-cis-epoxycarotenoid. The β-turn 7 BT7 (His81-Asp84) residue Phe83 is involved in hydrophobic interaction. To investigate the location and site of lipid oxidation and the mechanism which is unresolved [5,6], we introduced α-epoxycholesterol in the dioxygenase structure model. α-epoxycholesterol shares the same binding pocket as does the main substrate for dioxygenase 9-cis-epoxycarotenoid. However, α-epoxycholesterol induction in the 9-cis-epoxycarotenoid binding pocket brings about certain conformational changes which widen the base of the 9-cis-epoxycarotenoid binding pocket by displacing Arg67 a little and shifting Leu538 upward facilitating residues Ile471 and Arg406 to form a ridge around the closed end of 9-cis-epoxycarotenoid binding pocket. In the 9-cis-epoxycarotenoid binding pocket, 9-cis-epoxycarotenoid is anchored to the side walls by hydrophobic and weak polar interactions, while, α-epoxycholesterol is located in a hanging position by forming cross chain interactions with 9-cis-epoxycarotenoid and by forming hydrogen bonds with branched chain residue Val228; which may provide some stability by donating its backbone nitro group for the amidation of Glu409, that is bound to the O1 atom of 9-cis-epoxycarotenoid. The carbonyl group of Glu409 then dissociates as endogenous CO_2, which results in low pH, thus making the environment acidic essential for some important cellular functions. The acidic environment can be neutralized by Histidine residues which get protonated and also serve as a reason for an unusual pH dependence of the enzymatic functions. It is known that when two distinct ionizable groups participate in catalytic functions, a pH dependent activity have been observed in some specific cases. There are several proposed mechanisms for 2-3 ionizable groups generating two active forms of the enzyme, each catalyzed by a different catalytic efficiency. The residue on β-turn BT7 (His81-Asp84) Trp82 and Phe83 are interacting with the carbon atoms of α-epoxycholesterol. These interactions are hydrophobic and also provide some rigidity to the binding pocket conformations. Dioxygenase active site residues His225 and His339, located on the loop connecting between β-strands 16 (Arg328-Phe330) and 17 (Trp344-Glu346) interact with both substrates, however, the interaction of His225 is hydrophobic with 9-cis-epoxycarotenoid carbon atoms but with α-epoxycholesterol its nitrogen ND1 interacts with H1 atom. The nitrogen (NE2) atom of His339 is involved in polar interaction with the oxygen (O1) atom of α-epoxycholesterol. This suggests the possible mechanism of α-epoxycholesterol oxidation as the catalytic site residues His225, His339 and His529 along with other histidine residues (His165, His166) and Glu409, Gln537, Asp274 and Phe531 contribute to the three ionizable groups in the substrate binding site (Fig.2). The carbon and nitrogen atoms of His339 interact with the carbon atoms of 9-cisepoxycarotenoid while, it is forming a π-π interaction with the carbon atoms (C1, C5) of α-epoxycholesterol. The fourth active site residue His529 is located on the loop between β-sheets H and A, which connects β-strands 25(Gly486-His493) and 26(Val533-Thr534). The carbon and nitrogen atoms of His529 are interacting with carbon atoms of 9-cis-epoxycarotenoid, whereas, it is involved in a π-π interaction with carbon atoms (C1, C5) of α-epoxycholesterol. Other residues such as Phe404, Phe528 are also forming π-π interaction with carbon (C1, C5) atoms of α-epoxycholesterol. The residues that are involved in hydrophobic interactions with 9-cis-epoxycholesterol include Phe531 (CB-C9), Phe338 (CD2, CE2-C24), and the CG1 atom of β strand β20 (Val402-Val407) residue Val407 is interacting with C13 of 9-cis-epoxycarotenoid. These interactions indicate that His339 and His529 are involved in the nucleophillic attack and the final deprotonation step at His225; the residues Glu409, Gln537, Arg406, Thr160, Tyr33 and Asp274 are involved in the electron charge transfer in the process and in the rearrangement reactions, which are essential to accommodate both the substrate in the substrate binding pocket. These coordinated reactions are important steps in the activation process of dioxygenase for oxidative transformations of substrates and presumably, these reactions are essential for lipid and α-epoxycholesterol oxidation. The 9-cis-epoxycarotenoid alkane carbon-hydrogen bonds, carbon-oxygen bonds and the ortho- or 1,2-rearrangement on the α-epoxycholesterol aromatic ring is a possible mechanism for cis- carbon-carbon double bond cleavage in this class of enzymes. [5,6]

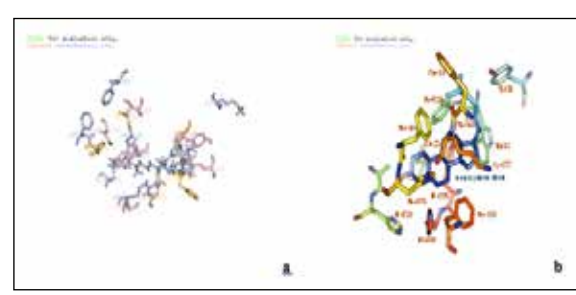

Figure 3, (a),(b) Interacting residues

The carbon atoms of the active site residue His225 can interact with the nitrogen and oxygen atoms of 9-cis-epoxycarotenoid. The residue on β-turn BT25 (Thr223-Pro226), Lys227 can interact with carbon atoms. The hydrogen atom H1 of Lys227 can also interact with the carbon atoms of 9-cis-epoxycarotenoid, while oxygen atom O2 of 9-cis-epoxycarotenoid can neutralize the ammonium group of Lys227 by interacting with carbon atoms, which can provide stability to the whole structure as well as 9-cis-

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epoxycarotenoid and α-epoxycholesterol binding pocket (Fig.2,3). The residue Arg406 located on β-strand β20 (Val402-Val407), which also contains a β-hairpin of 6 residues BH12 (Val402-Val407) of class 9:11 is contributing to the base of 9-cis-epoxycarotenoid and α-epoxycholesterol binding pocket. The carbon and nitrogen of Arg406 interact with carbon atom of 9-cis-epoxycarotenoid. Another residue Glu409 on β-turn BT44 (Asn408-Tyr411) interacts with oxygen atom O1 of 9-cis-epoxycarotenoid.

The electron withdrawing groups which, increase the acidity of the phenolic hydroxyl groups, reducing the catalytic activity but further cause reduction of Histidine residues His339 and His529 (Fig.2).[5,6] This can serve the basis for various metabolic functions which involve hydroxylation and methylation of substrates. In the dioxygenase-substrate complex, Tyr164 on binding with the 9-cis-epoxycarotenoid and α-epoxycholesterol is H-bonded with the anionic oxygen of the aromatic ring acting as a hydrogen donor, whereas, His339 is H-bonded with O1 atom and π-π interacting with carbon atoms of α-epoxycholesterol acting as a hydrogen acceptor.[5,6,9,10] Some formyl and nitro groups help deprotonation of the substrate and hence these groups increase the efficiency of the reaction, which serves the basis of modifying substrates by adding and substituting different groups in the main substrate aromatic ring.[5,6,9,10] The loss of hydroxyl group at position Tyr33 (its OH- group is interacting with C23 and C24 atoms of α-epoxycholesterol) and Tyr164 can be observed as increased substrate acidity and changed pH in the microenvironment, that can be linked to the pH dependent dioxygenase activity in the 9-cis-epoxycarotenoid cleavage and α-epoxycholesterol oxidation.[6] Mechanistically, the role of hydroxyl group can be that of binding the negative oxygen of the substrate, thus stabilizing the substrate-enzyme complex. The exposed epoxy groups of both substrates can be potential target for other epoxidases and pyruvate decarboxylase, hydrogenperoxide lyase, lipoxygenases and also dioxygenases which are supposed to be involved in metabolic processes of aldehyde, alcohol and esters. [5,11,12] In the abscisic acid biosynthesis the abscisic acid aldehyde xanthoxin is oxidized and subsequently gets converted into abscisic acid and the cholesteryl esters or oxidized cholesterol is transported by chylomicron and chylomicron remnants to both low density lipoprotein (LDL) and high density lipoprotein (HDL).[3,14-16] However, oxidized fatty acids have not been reported in LDL or HDL fractions at any time but studies indicate that α-epoxycholesterol transfer to LDL and HDL is mediated by cholesteryl ester transport protein from chylomicron or chylomicron remnants (CM/RM).[16]

Based on the dioxygenase-substrate structure complex, we can speculate that α-epoxycholesterol in the diet is incorporated into chylomicron or chylomicron remnant and also interacts with dioxygenase counterparts in humans such as tryptophan-2,3-dioxygenase, Indoleamine-2,3-dioxygenase, lipoxygenases, catechol dioxygenases and hydroxyphenylpyruvate dioxygenases and then transferred to LDL and HDL contributing to lipoprotein oxidation.[11-12] However, at which stage α-epoxycholesterol is oxidized by dioxygenases remains inconclusive as the structure complex does not provide insights into the functional biochemical consequences that can be observed by biochemical assays but here we are able to hypothesize that diet derived oxidized fatty acids in chylomicron remnants and oxidized cholesterol in remnants and LDL can accelerate atherosclerosis by increasing oxidized lipid concentrations in circulating LDL and chylomicron remnants.[3,14-16] The oxidized lipids or oxidized cholesterol rich diet can significantly increase lesions in aorta in patients with apo-E and LDL receptor gene mutations and also can contribute to the increased arterial atherosclerosis in their population.

The dioxygenase structure model offers into the very high affinity interaction with the main substrate 9-cis-epoxycarotenoid and will provide a template for understanding the location and mechanism of lipoprotein oxidation. The structure will provide a useful understanding of lipid or cholesterol oxidation and serve as an effective model for the development of dioxygenase as a potential target to incorporate chemical compounds as a substrate with better drug properties and the development of cholesterol lowering drugs and other related drugs to probe their biological roles.

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