

SHORT COMMUNICATION

Lipoxygenase Interactions With Natural Flavonoid, Quercetin, Reveal a Complex With Protocatechuic Acid in Its X-Ray Structure at 2.1 Å Resolution

Oleg Y. Borbulevych,¹ Jerzy Jankun,^{1,2} Steven H. Selman,^{1,2} and Ewa Skrzypczak-Jankun^{1*}¹Medical College of Ohio, Urology Research Center, Department of Urology, Toledo, Ohio²Medical College of Ohio, Department of Physiology and Molecular Medicine, Toledo, Ohio

ABSTRACT PUFA metabolites have a profound effect on inflammatory diseases and cancer progression. Blocking their production by inhibiting PUFA metabolizing enzymes (dioxygenases: cyclooxygenases and LOXs) might be a successful way to control and relieve such problems, if we learn to better understand their actions at a molecular level. Compounds with strong antioxidative and free radical scavenging properties, such as polyphenols, could be effective in blocking PUFA activities, and natural flavonoids possess such qualities. Quercetin belongs to the group of natural catecholic compounds and is known as a potent, competitive inhibitor of LOX. Structural analysis reveals that quercetin entrapped within LOX undergoes degradation, and the resulting compound has been identified by X-ray analysis as protocatechuic acid (3,4-dihydroxybenzoic acid) positioned near the iron site. Its C3-OH group points toward His523, C4-OH forms a hydrogen bond with O=C from the enzyme's C-terminus, and the carboxylic group is incorporated into the hydrogen bonding network of the active-site neighborhood via Gln514. This unexpected result, together with our previous observations concerning other polyphenols, yields new evidence about the metabolism of natural flavonoids. These compounds might be vulnerable to the co-oxidase activity of LOX, leading to enzyme-stimulated oxidative degradation, which results in an inhibitor of a lower molecular weight. *Proteins* 2004;54:13–19. © 2003 Wiley-Liss, Inc.

Key words: lipoxygenase; co-oxidase activity; lipoxygenase inhibitors; flavonoids; quercetin/metabolism/oxidative degradation

INTRODUCTION

LOXs are enzymes with a single iron cofactor (nonheme, no sulphur ligands), widespread in plant and various tissues of mammals,^{1,2} and mostly known for catalysis of lipid peroxidation. In humans, the products of unsaturated fatty acid metabolism are involved in inflammatory responses, heart diseases, cancerogenesis and cancer progres-

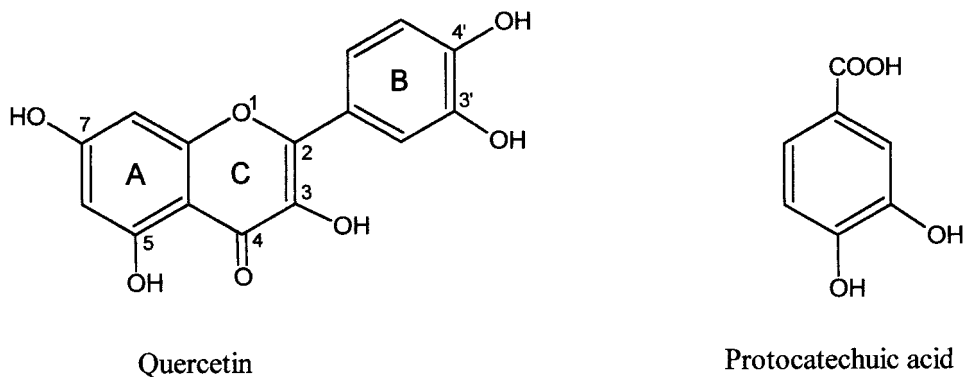
sion, and other diseases. Dietary PUFAs have an impact on cancer chemotherapy and radiation therapy.³ Studying structures and actions of LOX and PUFA metabolism via this pathway could be important in drug development. Recently attention has been paid to the preventative and medicinal value of dietary components, especially natural flavonoids with known inhibitory properties toward LOXs. All the molecules under consideration (curcumin, catechins, NGDA, etc., and their derivatives) possess one common component—a catecholic moiety. Their inhibitory effects on dioxygenases differ depending on the details of their structure (the number of aromatic rings, planarity, chirality, conjugated double bonds, etc.), the number of hydroxyls, and their positioning. Measured values of IC₅₀ could be at or below several tens of micromoles, which make them feasible candidates for therapeutics.⁴ Quercetin is the most abundant among the flavonoid molecules and can be found in the fruits, vegetables, seeds, nuts, and flowers of many plants.⁵ Its documented impact on human health includes cardiovascular protection; anticancer, antiviral, anti-inflammatory activities; antiulcer effects; and cataract prevention. Like other flavonoids, quercetin's

Abbreviations: CSD, Cambridge Structural Database; catechins, for example: EGCG, (–)-epigallocatechin gallate; EGC, (–)-epigallocatechin; quercetin, 3,3',4',5,7-pentahydroxyflavone [or 3',4',5,7-tetrahydroxyflavonol, or 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one]; catechol, o-dihydroxybenzene; curcumin, bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; DHB, 3,4-dihydroxybenzoic acid or protocatechuic acid; EDTA, ethylenediaminetetraacetic acid; EI, electron impact ionization; HFR, hydroxyl free radicals; 4HM, 4-hydroperoxy-2-methoxyphenol; LOX, lipoxygenase; soy LOX-3, soybean lipoxygenase isozyme 3; 4NC, 4-nitocatechol; NDGA, nordihydroguaiaretic acid; PUFA, polyunsaturated fatty acid; TLS, translation, libration and screw rotation.

Grant sponsor: American Diagnostica, Inc., Greenwich, CT; Grant sponsor: NIH; Grant number: CA90524; Grant sponsor: Frank D. Stranahan Endowment Fund for Oncological Research.

*Correspondence to: Ewa Skrzypczak-Jankun, Medical College of Ohio, Urology Research Center, Department of Urology, 3065 Arlington Avenue, Toledo, OH 43614. E-mail: eskrzypczak@mco.edu.

Received 29 April 2003; Accepted 7 July 2003



Scheme 1. Schematic representation of quercetin and protocatechuic acid.

beneficial influence seems to be related to its antioxidative and free radical scavenging capabilities, and inhibition of PUFA metabolism. On the other hand, besides dioxygenase activity, LOXs exhibit co-oxidase activity toward a wide range of chemicals. There is evidence that at least several hydrogen-donating organic molecules are co-oxidized by plant and mammalian LOXs.^{6,7} These observations lead to the conclusion that LOX is a versatile biocatalyst for biotransformation of endobiotics and xenobiotics.⁸ Understanding the peroxidative activity of LOX can help not only in regulating PUFA metabolism but also in predicting short- and long-term side effects and possible consequences of using this and other similar xenobiotics. This work presents the results of studying the interactions of soybean LOX-3 with quercetin by X-ray structural analysis of its molecular complex, and illustrates co-oxidative, LOX-catalyzed degradation of this flavonol (Scheme 1).

MATERIALS AND METHODS

Materials

Protein, LOX-3, was extracted from soybean seeds (*Resnick cultivar*), purified, and separated on the chromatofocusing column and crystallized according to a previously described protocol.⁹ A freshly obtained protein solution (10 mg/mL in Tris HCl, 0.1 M, pH 7) was mixed in a proportion of 3:6:1:2 with polyethylene glycol (PEG 8000, 20% w/v in citrate-phosphate buffer, 0.05 M, pH 4.6, 0.2% NaN₃ w/v), sodium phosphate buffer (0.1 M, pH 7), and deionized water, yielding a mixture with pH 5.3. "Standing drop" of 900 μ L of such solution was seeded with crushed crystals from the previous experiment and equilibrated against 3 mL of the above PEG solution. The crystals usually appear within a few days. Quercetin was purchased from Sigma (St. Louis, MO), dissolved in ethyl alcohol, and added to the crystallization medium as 4-fold in relation to the total molecular protein content, for soaking or co-crystallization. The final concentration of alcohol in the crystallization dish was kept within 0.8–1.2%.

X-Ray Analysis

Data for X-ray analysis were collected at room temperature by the oscillation method, on a Rigaku Raxis 4

dual-plate imaging detector, with a Cu rotating anode and focusing mirrors, at a crystal-to-detector distance of 140 mm, 2° oscillation angle, and an exposure time of 10 min per frame. For many reasons, one might see it as beneficial to collect the data at the cryogenic conditions; however, in the case of this crystal form, it turns out to be a disadvantage.¹⁰ The data were processed using the HKL software package¹¹ and merged separately for the crystals of each of the three different cases: (1) crystals soaked with quercetin for 15–30 h; (2) same as in case 1, but after 4 months, (3) crystals produced by co-crystallization. For all three cases, data collection was followed by calculations for a molecular replacement, a rigid body refinement, and the electron density maps. In all cases, a bulk positive peak in a difference electron density map was observed at 2 σ level next to the iron atom, giving us an opportunity to position there a molecule with at least one flat, six-membered ring with some substituents. There was no significant difference between maps, except that case 2 produced data of both lower resolution (3 Å) and quality. Data from case 1 showed the best statistics and completeness, and were used for all subsequent calculations and structure refinement. Ninety-one frames containing 232,374 reflections from three crystals were merged together, giving *R*-merge of 0.086 and 96.3% completeness for 52,289 reflections in the 68.0–2.1 Å resolution range. Programs Molrep and Refmac from CCP4 version 4.2.1 package¹² were used for molecular replacement and refinement, with anisotropic and bulk solvent corrections for the data. Prior to restrained refinement, TLS refinement¹³ using one TLS group had been performed. TLS parameters obtained were included in all subsequent steps of refinement. Graphical evaluation of the model, fitting to the maps, and positioning of the water molecules were performed utilizing Xtal-View¹⁴ or CHAIN version 7.2.¹⁵ Final results are summarized in Table I and deposited in the Protein Data Bank (PDB entry 1N8Q).

Molecular Modeling, Statistical and Theoretical Calculations

Molecular modeling calculations were performed (1) to test the flexibility of the quercetin C ring and its alignment with the other two aromatic moieties, and (2) to find the

TABLE I. Data Collection and Refinement Statistics

Data: Space group and unit cell dimensions	
C2, a = 112.6, b = 137.0, c = 61.8 Å, β = 95.5°	
Resolution limits (Å)	50.0–2.1
Unique reflections	52281
Completeness overall, last shell 2.18–2.10 Å (%)	96.3, 86.2
R_{merge} overall, last shell 2.18–2.10 Å	0.07, 0.45
Refinement (CCP4):	
Protein, inhibitor, water (nonhydrogen atoms only)	6779,11,463
R (all reflections)	0.192
R (working set, 49,622 reflections)	0.189
R_{free} (validation set, 2659 reflections)	0.245
RMS deviation from ideal geometry	
Bond lengths (Å)	0.016
Bond angles (°)	2.05
Dihedral angles (°)	7.58

most probable conformation based on the known small-molecule structures. *Ab initio* calculations (1) were performed using GAMESS program.¹⁶ Statistical evaluation of the structures deposited in the CSD (2) were performed using programs from CSD version 5.24.¹⁷

RESULTS AND DISCUSSION

Molecular modeling of the LOX structure incorporating quercetin imposes changes in several segments of the enzyme's structure. In the case of the soybean LOX-3, placing quercetin in the central cavity requires movement of Gln514 and a large shift in the position of Leu277, Ile557, and Leu773. The quercetin molecule fits best if oriented with O5 near O857, O2 (=C) above Fe and O3', O4' colliding with Gln514. Quercetin can adapt to the shape of the available space better if a planarity of the C ring is not strictly obeyed. The quantum-chemical calculations and optimization of the quercetin molecule at the HF/6-31G** level of theory leads to a structure with a planar C ring and the B ring rotated with respect to the plane of A,C rings by -23.4° . Despite the fact that the C ring has the planar equilibrium conformation, the conformational behavior of this ring is quite peculiar. Previously it was found¹⁸ that the 4*H*-pyran-4-one ring possesses a high conformational flexibility according to HF/6-31 calculations. Namely, the transition from the planar equilibrium conformation to a boat with the $C_{\text{sp}2}=C_{\text{sp}2}-C(=O)-C_{\text{sp}2}$ torsion angle $\pm 10^\circ$ gives rise to an increase of the total energy of the molecule less than 0.56 kcal/mole. In the case of 4*H*-pyran-4-one, its conformational flexibility is caused by the presence of two groups of opposing influence that determine geometry of the ring. The first group involves a 1,2 allylic strain and a conjugation, which favors a planar conformation. The second group includes a bending strain and a nonaromatic (7 π -electron) system, which favors a nonplanar geometry. As a result, the overall energy change is small under relatively large changes of the relevant torsion angles. Moreover, in quercetin, the 4*H*-pyran-4-one moiety is annealing with a benzene ring giving an A,C conjugated rings system. Such annealing usually leads to a further increase in the conforma-

tional flexibility of a dihydrocycle.¹⁹ Our statistical analysis performed for the 4*H*-chromen-4-one fragment (A,C ring without substituents) and based on 160 structures from CSD version 5.24,¹⁷ shows that the $C_{\text{sp}2}=C_{\text{sp}2}-C(=O)-C_{\text{Ar}}$ torsion angle can vary up to 10° . Such conformational flexibility of the C ring in quercetin might be of importance for this molecule to make adjustments to the steric constraints and/or spatial requirements for suitable conformation/orientation during a catalytic reaction, should quercetin undergo an oxidative degradation to a smaller molecule.

X-ray analysis and calculated maps have not provided evidence for a presence of the intact natural flavonoid used for the soaking experiment. Lack of electron density that could accommodate a *whole* quercetin molecule prompted us to examine the properties of quercetin and how they might have performed in our experimental conditions. Quercetin is a flavonol that can be easily oxidized in an aqueous environment, and in the presence of iron and hydroxyl free radicals.^{20–25} All these factors were present in our experiment. It is well known that X-ray radiation can generate free radicals in protein crystals, including hydroxyl radicals. In addition, as in many crystallization protocols, NaN_3 was used in our crystallization media. Therefore, there is a chance for a presence of the azide radical, which is known to induce phenoxyl radicals, while in the company of the catechol-like molecules.²³ Studies of different oxidized flavonols by mass spectroscopy²⁰ have shown no $[M+]$ as a base peak due to loss of aromaticity of the C ring. The most prominent fragment in electron impact (EI) mass spectra of an oxidized quercetin is $m/z = 154$, corresponding to dihydroxybenzoic acid. HFR-mediated oxidative degradation of quercetin described by other researchers²⁵ suggests 2,4,6-trihydroxybenzoic acid as another hypothetical possibility. There is no proven mechanism for this transformation, but considering our experimental conditions (aerobic, aqueous media, presence of the Fe ion, traces of alcohol, an acidic pH), we are inclined to agree with the mechanism of quercetin oxidation proposed by Makris and Rossiter²⁵ (mediated by the metal ion and the HFRs). Another mechanism proposed by Krishnamachari et al.²¹ asks for the incorporation of an oxygen molecule leading to peroxide. Available experimental data confirm a covalent linkage, Enz-Fe-O-O-R in the "purple" state of LOX, and such intermediates were successfully reported for soy LOX-3 complexes with (i) 13-(S)-hydroperoxy-9,11-(*cis,trans*)-octadecadienoic acid and (ii) 4-hydroperoxy-2-methoxyphenol (PDB entries 1IK3, 1HU9^{26,27}). These peroxides were either introduced into the crystals (i) or induced in the crystal by photoreaction (ii). In both cases, the characteristic purple color was visible and lasted a few hours. In the presently reported complex, no color change was noticed. Thus, it is probable that this reaction is either too fast to notice, occurs by electron donation (possibly $\text{Fe}^{+2} - e^- \rightarrow \text{Fe}^{+3}$) as in other cases in aqueous media,²³ or is radical mediated. Another interesting documentation comes from the experiment describing the fate of quercetin in the cultured human hepatocarcinoma cell line, Hep G2.²⁸ The authors observed a rapid elimination of the flavonoid and no unchanged compound present beyond 8 h. The metabolites

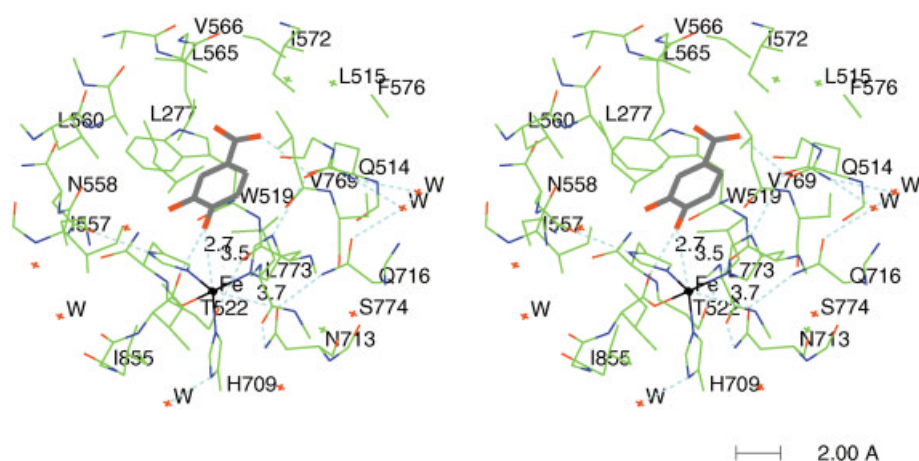


Fig. 1. Stereo drawing of LOX-3:protocatechuic acid (gray) within 10 Å radius, where W stands for the water molecules. Chosen hydrogen bonds depicted as a blue dashed lines.

were mainly products of oxidative degradation, with the major one identified as protocatechuic acid. The degradation of quercetin was very rapid in the presence of hydrogen peroxide and ferrous chloride, indicating a Fenton reaction. It was also noticed that part of that degradation pathway in the cell media could be inhibited by EDTA, which is a well-known chelator for metals. Our X-ray measurements were done after 15 h of soaking LOX crystals with quercetin. Fe^{+2} is a natural cofactor in LOXs, whose biological activity can go via Fenton reaction. All these observations prompted our conclusion that the electron density map corresponds to the product of oxidative degradation and not quercetin itself.

After careful consideration of the possible molecules (3,4-dihydroxy- vs 2,4-dihydroxy-, and 2,4,6-trihydroxybenzoic acid), protocatechuic acid (Scheme 1) has proven to be the best fit, and such a model was refined against the experimental data. The coordinates and structure factors, deposited in the PDB entry 1N8Q, correspond to amino acids 9–857, one Fe^{+2} , protocatechuic acid, and 463 water molecules (Table I). The orientation of the small molecule in the enzyme's central cavity is illustrated in Figure 1. The iron ion is covalently bound to four ligands: NE2 atoms from His 518, 523, and 709, and to the carboxylic OXT from the terminus Ile857. However, its coordination arrangement resembles a distorted octahedron with Asn713 (OD1...Fe of 3.65 Å) and protocatechuic acid (O4...Fe of 3.48 Å) in a nonbinding distance, but in a proper geometric position to fulfill the octahedral coordination of six ligands. The average values for the coordination sphere are Fe–ligand = 2.12(3) Å, $90 \pm 10^\circ$ and $164 \pm 8^\circ$ for “90” and “180” degree angles, respectively, with N–Fe–N = $97(6)^\circ$, N–Fe–O = $86(11)^\circ$, and O–Fe–O = $92(9)^\circ$, where “–” describes either binding or nonbinding distance. Analysis of 239 structures from the CSD^{29,30} containing Fe with 3 N and 3 O ligands provide the following ranges of angular values: N–Fe–N = $74 \div 80^\circ$ or $164 \div 182^\circ$, N–Fe–O = $85 \div 100^\circ$ or $165 \div 180^\circ$, O–Fe–O = $94 \div 100^\circ$ or $160 \div 178^\circ$. Distortions from “90” and “180” are therefore expected. Although the nitrogen side of the coordination sphere seems to be slightly flatter compared to the native enzyme (PDB entry

1LNH), the observed values are within 2σ , so more data are needed for such molecular LOX complexes to determine if this is a common trend.

The 3,4-dihydroxybenzoic acid molecule positions itself with the hydroxyls toward the carboxylic group of Ile857, and the carboxyl between Gln514 and Trp519. The OH group in the *para* position forms a strong hydrogen bond O4...OXT Ile857 of 2.7 Å, and carboxylic group makes a weak bond O2...OE2 Gln514 of 3.2 Å. Two other oxygen atoms present in this molecule are anchored by C–H...O bonds: O3(*meta* OH)...H-CE1 His523 of 2.7 Å, and O1...H-CG2 Val566 of 3.0 Å. The plane of the carboxylic group is rotated by 30.6° in relation to the aromatic ring. The iron ion and this nearby small molecule in the complex reported here, show elevated temperature factors, $B_j = 45$ and 62Å^2 , respectively, in contrast to $\sim 30 \text{Å}^2$ (Ile857 and His518) and $\sim 20 \text{Å}^2$ (His523, 709, Asn713) observed for other residues near iron. This relationship between the occupancy and the temperature factor of the iron ion is well illustrated in soy LOX-1 and its mutants,³¹ where measurements were done at 100 K and the iron content was known. Elevated temperature factors were observed for the inhibitor in complex with rabbit reticulocyte 15-LOX (PDB entry 1LOX³²), and this might reflect the fact that a crystallographer seldom knows the exact stoichiometry of such complexes and the occupancy factor can easily be misjudged. The iron content in LOX could be less than 1.0,^{31,33} especially in the presence of chelating metals. Quercetin is a known iron chelator and can form a 2:1 complex. It is therefore possible that, in our experiment, the iron content in LOX could be somewhat depleted, and less than in the wild enzyme with which we have started. The occupancy for the inhibitor included in the model was arbitrarily assigned as 0.5 based on the similarity of this compound to the known structure of LOX-3:4-nitrocatechol complex, where stoichiometry was measured by microcalorimetric titration.³⁴ Refinement of the occupancies for both iron and protocatechuic acid in SHELXL program³⁵ returned the values 0.66 and 0.48, respectively.

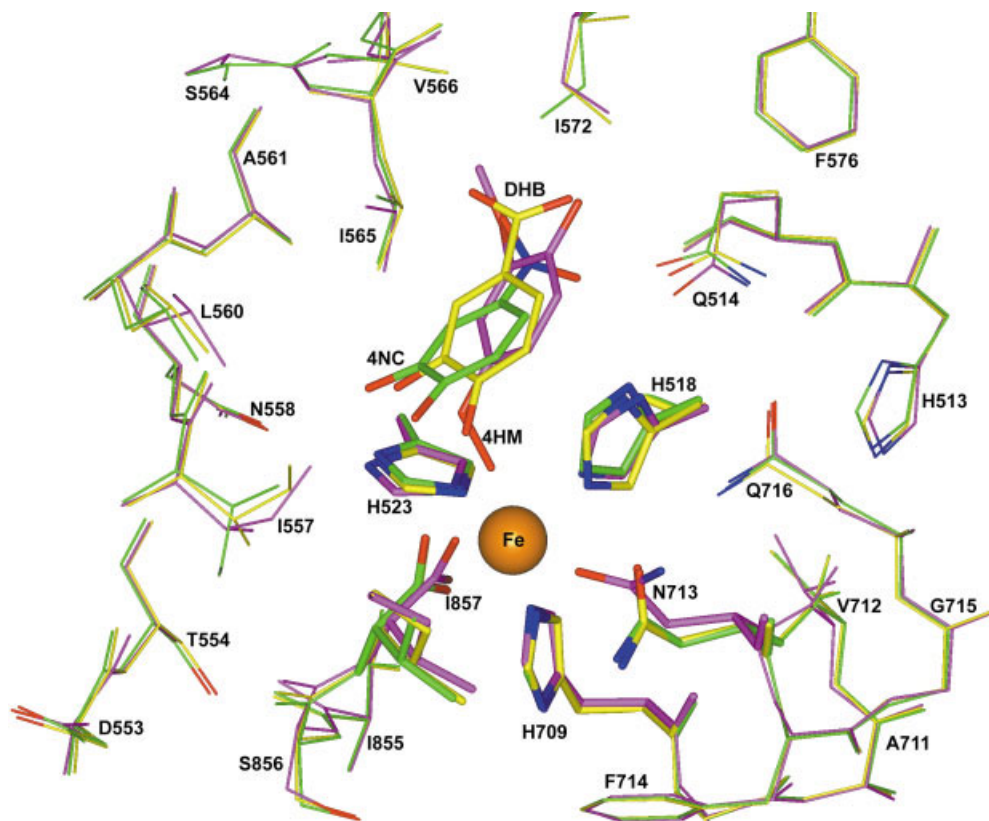


Fig. 2. Superimposition of the three complexes of soy LOX-3 with catechol derivatives: protocatechuic acid (DHB, present study, yellow), 4-nitrocatechol (4NC, green) and 4-hydroperoxy-2-methoxyphenol (4HM, purple). Selected residues illustrate a “slice” of the structure in the vicinity of the Fe active site; the other residues and water molecules have been omitted for clarity.

It confirms our interpretation, but due to inadequate resolution (for this program) and the data to variable ratio, we decided not to pursue the refinement within this program. The protocatechuic acid binds in the same location as 4-nitrocatechol (PDB entry 1NO3) and the product of curcumin photodegradation, 4-hydroperoxy-2-methoxyphenol (PDB entry 1HU9). These three complexes are superimposed in Figure 2. Our observations from this and the previously reported experiments indicate that curcumin and quercetin in presence of LOX can undergo degradation to the catechol derivatives. Such molecules, either introduced as is (4NC), or as the products of LOX catalyzed reaction (4HM and DHB), bind to this enzyme in the same space and in a similar way (Figs. 2 and 3). The lining of the cavity where these inhibitors bind is shown in Figure 3, with the surface colored according to the lipophilicity potential. The protein molecule in Figure 3 corresponds to the DHB complex, the inhibitors DHB, 4NC and 4HM are colored as in Figure 2. A major difference is noticeable between the peroxy-complex (4HM) and the other two, in the movement of oxygen-providing ligands (Fig. 2). This is related to the change of the iron status ($\text{Fe}^{+2} \rightarrow \text{Fe}^{+3}$) in the “purple” form of LOX (Enz-Fe-O-O-R).

Like curcumin and quercetin, protocatechuic acid is a natural compound present in onion, red and white cab-

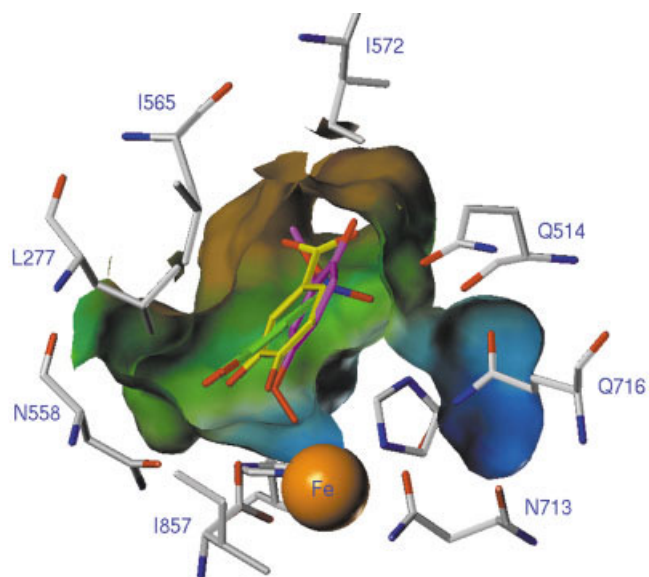


Fig. 3. Lining of the cavity with inhibitors DHB, 4NC, and 4HM (same colors as in Fig. 2), protein from 1N8Q; surface colored according to the lipophilicity potential, where brown corresponds to the highest hydrophobic area, and blue to the highest hydrophilic area of the molecule [SYBYL version 6.9 (Tripos, Inc., St. Louis, MO) calculates it according to the method of Audry et al., Eur J Med Chem 1986;21:71–72].

bage, and other plants. It is a substrate for catecholic dioxygenases catalyzing the ring-opening reaction of protocatechuate and related compounds. The finding that LOX can turn different compounds into simple catechol derivatives (with one aromatic ring only) might be of importance as an additional small piece of a "jigsaw puzzle" in the much bigger picture of drug metabolism.

Co-oxidative activity of LOX and comments on its kinetic behavior: LOXs are mostly known for their peroxidase activity and metabolizing unsaturated fatty acids. Their co-oxidase activity is much less explored. Known experimental results provide evidence for LOX-catalyzed reactions, such as oxidation, hydroxylation, epoxidation, sulfoxidation, desulfuration, dearylation, and N-dealkylation. Kulkarni⁸ proposes four possible mechanisms of such LOX co-oxidative activity: (1) hydroperoxide dependent reactions, (2) peroxy radical-mediated reactions, (3) electron transfer-dependent reactions, and (4) other reactions, where a small-molecule substrate is oxidized in the absence of exogenous PUFA(s) or peroxide(s). Each case requires a stimulus to initiate a change in the iron valency, from Fe⁺² in the native enzyme into an active form with Fe⁺³ co-factor. In our experiment, no fatty acid or peroxide was added to the crystallization media to which quercetin was introduced. However, it has been found that quercetin can rapidly produce H₂O₂ in cell culture media,³⁶ so one may hypothesize that this *in vitro* process could have been simulated in our experiment. Also, what we observe in X-ray analysis results from data collected using an ionizing radiation, so the presence of the radiation-induced radicals cannot be excluded.

The results from X-ray analysis agree with other observations made so far about LOX as a versatile biocatalyst for biotransformation of endobiotics and xenobiotics (see review articles^{3,37}). It might also provide some hints as to why the kinetic studies of LOX-3 inhibition by quercetin³⁸ show competitive and linear behavior on a Lineweaver-Burke plot, but only to a ~2 μM concentration, and at 2.5 μM it becomes nonlinear. This corresponds to LOX activity of ~60%. Similar observations have been made for other polyphenols, such as curcumin, EGCG and EGC,³⁸ and may indicate that the inhibition is a mechanism of several steps, where binding and co-oxidative degradation of a given polyphenolic compound is followed by inhibition of LOX by the resulting, smaller molecule. The complexity of LOX kinetics have been discussed by many researchers. Gibian and Galaway, in 1976, provided an early, detailed description for pure isozyme soybean LOX-1.³⁹ Ludwig et al., in 1987, described results for kinetic studies of mammalian, rabbit reticulocyte LOX.⁴⁰ Both models suggest a complex, multistep mechanism, and in both cases, the studies were performed using a natural substrate, linoleic acid. That mechanism gets more complicated when one considers interactions of LOX with small-molecule inhibitors. Walther et al., in 1999, pointed out that a course of inhibition (noncompetitive vs competitive) and its reversibility depend on the oxidation state of iron (i.e., whether the enzyme is catalytically silent with Fe⁺² or preoxidized and active with Fe⁺³).⁴¹ In their studies of rabbit reticulo-

cyte 15-LOX inhibition by ebselen, catalytically silent ground-state LOX was irreversibly inactivated at nanomolar ebselen concentrations, but the catalytically active enzyme form was only competitively inhibited in the lower micromolar range. Our kinetic data cited above concern the active state of soy LOX-3 enzyme. The *in vivo* susceptibility of LOXs to inhibitors may therefore depend not only on the source of LOX and its isozyme^{34,42} but also on the oxidation state of iron and the competition between peroxidase and co-oxidase activities of enzyme.

CONCLUSIONS

Catecholic compounds play an important role in reducing the oxidative stress *in vivo*, and this property is claimed in the literature to be responsible for their anti-inflammatory and antitumorigenic properties. The exact nature of the chemistry involved in the experiment reported here itself presents an interesting puzzle for further cautious examination by methods other than X-ray analysis. Both compounds (i.e., quercetin and protocatechuic acid), have many common biological activities, antioxidant and antitumor, among many others. According to the Phytochemical Database,⁴ protocatechuic acid is a better antioxidant and requires a 2/3 dosage of quercetin. Recent clinical studies related to cancer research^{43,44} did not confirm that being an effective antioxidant is a sufficient criterion for the natural compound to have chemopreventive and anticancer properties. These two trials have caused a rethinking of the use of natural compounds as chemoprevention agents utilizing the antioxidant concept. It is therefore likely that these chemicals (natural flavonoids among them) take actions that disrupt a specific pathway or inhibit enzymes important in cancerogenesis. Their interactions with LOX can be more complicated than simply blocking the access to the enzyme's active site. Our studies on LOX and quercetin contribute to the understanding of biocatalytic properties of this enzyme and its role in the metabolism of this popular (as a medicinal remedy) flavonol and possibly other, similar compounds.

ACKNOWLEDGMENTS

This work was supported in part by grants from: American Diagnostica Inc., Greenwich, CT, NIH CA90524, and the Frank D. Stranahan Endowment Fund for Oncological Research.

REFERENCES

1. Yamamoto S. Mammalian lipoxygenases: molecular structures and functions. *Biochim Biophys Acta* 1992;1128(2-3):117-131.
2. Siedow JN. Plant lipoxygenase: structure and function. *Annu Rev Plant Physiol Plant Mol Biol* 1991;42:145-188.
3. Conklin KA. Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation. *Altern Med Rev* 2002;7:4-21.
4. Duke J. *Phytochemical Database*. Beltsville, MD: USDA-ARS-NGRL; April 2003.
5. Monograph: quercetin. *Altern Med Rev* 1998;3:140-143.
6. Kulkarni AP, Sajan MP. A novel mechanism of glutathione conjugate formation by lipoxygenase: a study with ethacrynic acid. *Toxicol Appl Pharmacol* 1997;143:179-188.
7. Datta K, Sherblom PM, Kulkarni AP. Co-oxidative metabolism of 4-aminobiphenyl by lipoxygenase from soybean and human term placenta. *Drug Metab Dispos* 1997;25:196-205.
8. Kulkarni AP. Lipoxygenase—a versatile biocatalyst for biotrans-

- formation of endobiotics and xenobiotics. *Cell Mol Life Sci* 2001;58:1805–1825.
- Skrzypczak-Jankun E, Amzel LM, Kroa BA, Funk MO Jr. Structure of soybean lipoxygenase L3 and a comparison with its L1 isoenzyme. *Proteins* 1997;29:15–31.
 - Skrzypczak-Jankun E, Bianchet M, Amzel LM, Funk MO. Flash-freezing causes a stress induced modulation in a crystal structure of soybean lipoxygenase L3. *Acta Crystallogr D Biol Crystallogr* 1996;52:959–965.
 - Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol A* 1997;276:307–326.
 - The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* 1994;50:760–763.
 - Winn MD, Isupov IM, Murshudov GN. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr D Biol Crystallogr* 2001;57:122–133.
 - McRee DE. XtalView/Xfit—a versatile program for manipulating atomic coordinates and electron density. *J Struct Biol* 1999;125:156–165.
 - Sack JS. CHAIN—a crystallographic modeling program. *J Mol Graph* 1988;6:224–225.
 - Schmidt MWB, Baldridge KK, Boatz JA, Elbert ST, Gordon MS, Jensen JH, Koseki S, Matsunaga N, Nguyem KA, Su SJ, Windus TL, Dupuis M, Montgomery JA. The general atomic and molecular electronic structure system. *J Comput Chem* 1993;14:1347–1363.
 - Allen FH, Motherwell WD. Applications of the Cambridge Structural Database in organic chemistry and crystal chemistry. *Acta Crystallogr B* 2002;58:407–422.
 - Shishkin OV. Conformational flexibility of six-membered 1,4-dihydrocycles. *J Mol Struct* 1997;412:115–120.
 - Shishkin OV. Conformational flexibility of six-membered dihydrocycles. *Russ Chem Bull* 1997;46:1981–1991.
 - Jungbluth G, Ruhling I, Ternes W. Oxidation of flavonols with Cu(II), Fe(II) and Fe(III) in aqueous media. *J Chem Soc Perk Trans 2* 2000;9:1946–1952.
 - Krishnamachari V, Levine LH, Pare PW. Flavonoid oxidation by the radical generator AIBN: a unified mechanism for quercetin radical scavenging. *J Agric Food Chem* 2002;50:4357–4363.
 - Afanasev IB, Dorozhko AI, Brodskii AV, Kostyuk VA, Potapovitch AI. Chelating and free radical scavenging mechanism of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* 1989;38:1763–1769.
 - Jovanovic SV, Steenken S, Hara Y, Simic MG. Reduction potentials of flavonoid and model phenoxy radicals: Which ring in flavonoids is responsible for antioxidant activity? *J Chem Soc Perk Trans 2* 1996;11:2497–2504.
 - Dangles O, Fargeix G, Dufour C. One-electron oxidation of quercetin and quercetin derivatives in protic and non protic media. *J Chem Soc Perk Trans 2* 1999;7:1387–1395.
 - Makris DP, Rossiter JT. Hydroxyl free radical-mediated oxidative degradation of quercetin and morin: a preliminary investigation. *J Food Comp Anal* 2002;15:103–113.
 - Skrzypczak-Jankun E, Bross RA, Carroll RT, Dunham WR, Funk MO Jr. Three-dimensional structure of a purple lipoxygenase. *J Am Chem Soc* 2001;123:10814–10820.
 - Skrzypczak-Jankun E, McCabe NP, Selman SH, Jankun J. Structure of curcumin in complex with lipoxygenase and its relation to cancer. *Int J Mol Med* 2003;12:17–24.
 - Boulton DW, Walle UK, Walle T. Fate of the flavonoid quercetin in human cell lines: chemical instability and metabolism. *J Pharm Pharmacol* 1999;51:353–359.
 - Bruno IJ, Cole JC, Edgington PR, Kessler M, Macrae CF, McCabe P, Pearson J, Taylor R. New software for searching the Cambridge Structural Database and visualizing crystal structures. *Acta Crystallogr B* 2002;58:389–397.
 - Allen FH. The Cambridge Structural Database: a quarter of a million crystal structures and rising. *Acta Crystallogr B* 2002;58:380–388.
 - Tomchick DR, Phan P, Cymborowski M, Minor W, Holman TR. Structural and functional characterization of second-coordination sphere mutants of soybean lipoxygenase-1. *Biochemistry* 2001;40:7509–7517.
 - Gillmor SA, Villasenor A, Fletterick R, Sigal E, Browner MF. The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. *Nat Struct Biol* 1997;4:1003–1009.
 - Knapp MJ, Seebeck FP, Klinman JP. Steric control of oxygenation regiochemistry in soybean lipoxygenase-1. *J Am Chem Soc* 2001;123:2931–2932.
 - Pham C, Jankun, J, Skrzypczak-Jankun, E, Flowers, RA, Funk, MO. Structural and thermochemical characterization of lipoxygenase-catechol complexes. *Biochemistry* 1998;37:17952–17957.
 - SHELXTL-NT, Version 6.12. Madison, WI: Bruker AXS Inc.; 2001.
 - Long LH, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Biophys Res Comm* 2000;273:50–53.
 - Kulkarni AP. Role of biotransformation in conceptual toxicity of drugs and other chemicals. *Curr Pharm Des* 2001;7:833–857.
 - Skrzypczak-Jankun E, Zhou K, McCabe NP, Kernstock R, Selman SH, Funk MO Jr., Jankun J. Natural polyphenols in complexes with lipoxygenase—structural studies and their relevance to cancer. San Francisco: AACR; 2002. p 205.
 - Gibian MJ, Galaway RA. Steady-state kinetics of lipoxygenase oxygenation of unsaturated fatty acids. *Biochemistry* 1976;15:4209–4214.
 - Ludwig P, Holzthutter HG, Colosimo A, Silvestrini MC, Schewe T, Rapoport SM. A kinetic model for lipoxygenases based on experimental data with the lipoxygenase of reticulocytes. *Eur J Biochem* 1987;168:325–337.
 - Walther M, Holzthutter HG, Kuban RJ, Wiesner R, Rathmann J, Kuhn H. The inhibition of mammalian 15-lipoxygenases by the anti-inflammatory drug ebselen: dual-type mechanism involving covalent linkage and alteration of the iron ligand sphere. *Mol Pharmacol* 1999;56:196–203.
 - Schewe T, Kuhn H, Rapoport S. Positional specificity of lipoxygenases and their suitability for testing potential drugs. *Prosta Leukotr Med* 1986;23:155–160.
 - Goodman GE. Prevention of lung cancer. *Crit Rev Oncol Hematol* 2000;33:187–197.
 - Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. Flavonol and flavone intake and the risk of cancer in male smokers (Finland). *Cancer Cause Control* 2001;12:789–796.