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REVIEW

Applications of Stereospecifically-Labeled Fatty Acids in Oxygenase and Desaturase Biochemistry

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Abstract Oxygenation and desaturation reactions are inherently associated with the abstraction of a hydrogen from the fatty acid substrate. Since the first published application in 1965, stereospecific placement of a labeled hydrogen isotope (deuterium or tritium) at the reacting carbons has proven a highly effective strategy for investigating the chemical mechanisms catalyzed by lipoxygenases, hemoprotein fatty acid dioxygenases including cyclooxygenases, cytochromes P450, and also the desaturases and isomerases. This review presents a synopsis of all published studies through 2010 on the synthesis and use of stereospecifically labeled fatty acids (71 references), and highlights some of the mechanistic insights gained by application of stereospecifically labeled fatty acids.

Keywords Lipid biochemistry · Lipoxygenase · Cyclooxygenase · Cytochrome P450 · Desaturase · Eicosanoids · Oxylipins

Introduction

In 1965, Schroepfer and Bloch reported the preparation of four different stearic acids containing a single stereospecific D or L tritium label on either the 9- or 10-carbon and their use in analyzing the mechanism of desaturation to oleic acid [1]. Other applications in unraveling mechanisms of fatty acid transformation soon followed, and many of the fundamental concepts in fatty acid, eicosanoid or oxylipin biosynthesis rest squarely on the results of this type of experiment. The heart of this review is a chronological list of all the primary literature we could find that used stereospecifically labeled substrates to study mechanisms of fatty acid oxygenation, desaturation, or double bond isomerization (Refs. 1–71, Tables 1, 2). The approach was recognized as a powerful tool back in the 1960s and it continues to be fruitfully applied, with about one third of our list of citations being published from the year 2000 onwards. Although there are numerous additional applications in which stereoselective hydrogen abstraction is measured from other types of substrate, for example in the cytochrome P450 field, this review is limited to fatty acid-related biochemistry. We also do not include purely regiospecific labeling experiments (e.g. using [11,11-$^2H_2$] linoleic acid).

Mechanistic Insights in Oxygenase and Desaturase Reactions: General "Rules"

Insights gained from an understanding of the stereoselectivity of hydrogen abstraction are a spatial understanding of the biochemical transformation, and from isotope effects, the order of events. Among the fundamental mechanisms established using this methodology is the almost universal "rule" in cyclooxygenase and lipoxygenase catalysis that reaction is initiated by a stereospecific hydrogen removal from the CH_2 between two *cis* double bonds, and that molecular oxygen is covalently coupled on the opposite face of the reacting fatty acid—the so-called antarafacial

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Table 1 Use of stereospecifically-labeled fatty acids in mechanistic studies on oxygenases

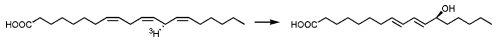
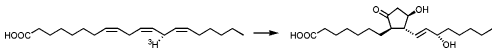
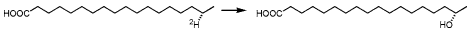
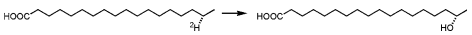
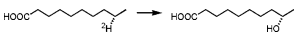
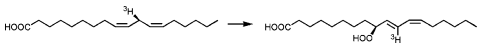
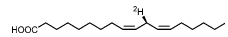
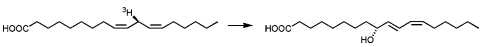
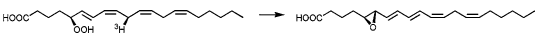
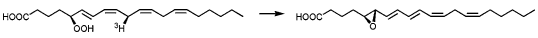
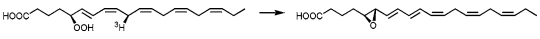
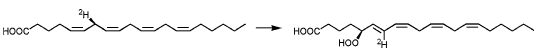
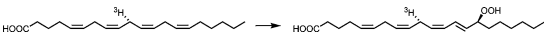
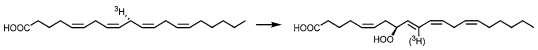
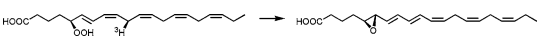
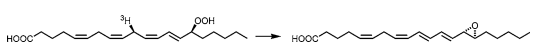
Author(s)	Year	Fatty acid	Label	Reaction (Enzyme)	Reference	Reaction, or synthetic product
Hamberg & Samuelsson	1967	20:3ω3	13-^{3}H	Soybean L-1 (15-LOX)	[2]	
Hamberg & Samuelsson	1967	20:3ω3	13-^{3}H	COX-1 (RSVM)	[3]	
Hamberg & Samuelsson	1967	20:3ω3	13-^{3}H	COX-1 (RSVM)	[4]	
Morris & Hitchcock	1968	16:0	2-^{3}H	α-hydroxylation, pea leaves	[5]	
Jones	1968	18:0	17-^{3}H	ω-1 hydroxylation, yeast	[6]	
Heinz et al.	1969	18:0	17-^{3}H	ω-1 hydroxylation, yeast	[7]	
Hamberg & Björkhem	1971	10:0	9-^{2}H	ω-1 hydroxylation, rat liver	[8]	
Egmond et al.	1972	18:2ω6	11-^{3}H	Soybean 13-LOX, corn 9-LOX	[9]	
Egmond et al.	1973	18:2ω6	11-^{3}H	Synthesis of label	[10]	
Hamberg & Samuelsson	1980	18:2ω6	11-^{3}H	COX-1 (RSVM)	[11]	
Hamberg & Hamberg	1980	20:4ω6	10-^{3}H	Platelet 12-LOX	[12]	
Panossian et al.	1982	20:4ω6	10-^{3}H	PMN 5-LOX	[13]	
Maas et al.	1982	20:4ω6	10-^{3}H	PMN 5-LOX, platelet 12-LOX	[14]	
Hammarstrom	1983	20:5ω3	10-^{3}H	Mastocytoma cells, 5-LOX	[15]	
Maas & Brash	1983	20:4ω6, 15-HPETE	10-^{3}H	Platelet, leukocyte 12-LOX	[16]	
Corey & Lansbury	1983	20:4ω6	7-^{2}H	Rat 5-LOX, potato 5-LOX	[17]	
Maas et al	1985	20:3/20.4 15-HPETE	10-^{3}H	Synthesis of label, soybean L-1	[18]	
Brash et al.	1985	20:4ω6, 15-HPETE	10-^{3}H	Autoxidation of 20.4ω6, 15-HPETE	[19]	
Brash et al.	1986	20:4ω6	10-^{3}H	Platelet 12-LOX	[20]	
Shimizu et al.	1986	5-HPETE	10-^{3}H	Murine mast cell 5-LOX	[21]	
Ueda et al.	1986	20:4ω6	10-^{3}H	Porcine 5-LOX	[22]	
Hawkins & Brash	1987	**20:5ω6**	10-^{3}H	Sea urchin 12R-LOX	[23]	
Brash et al.	1989	20:4/15-HPETE	10-^{3}H	Porcine leukocyte 12-LOX	[24]	

Table 1 continued

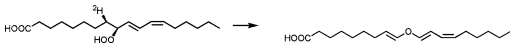
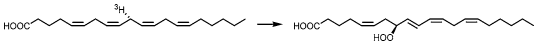
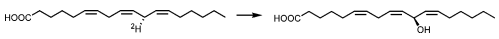
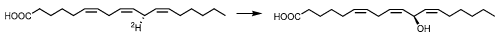
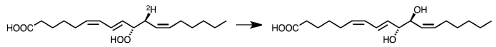
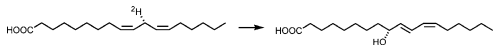
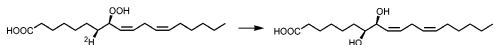
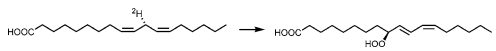
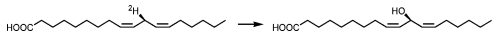
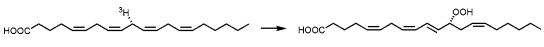
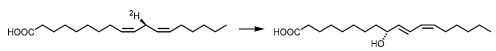
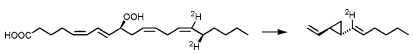
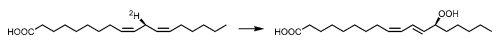
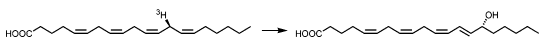
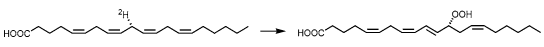
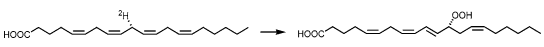
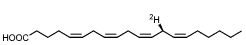
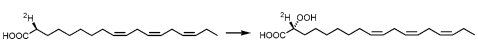
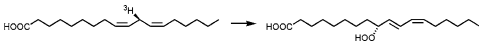
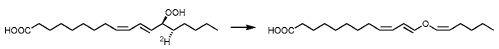
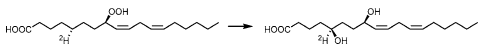
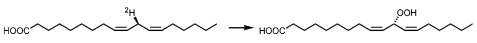
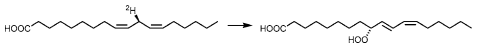
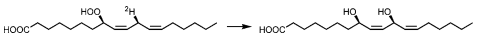
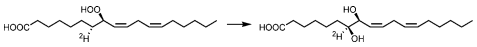
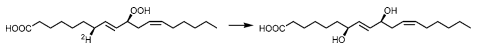
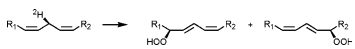
Author(s)	Year	Fatty acid	Label	Reaction (Enzyme)	Reference	Reaction, or synthetic product
Fahlstadius et al.	1990	9-HPODE	8-^{2}H	Potato divinyl ether synthase	[25]	
Hughes & Brash	1991	20:4ω6	10-^{3}H	Coral 8R-LOX, mouse 8S-LOX	[26]	
Hamberg	1992	18:3ω6	8-^{2}H, 11-^{2}H	Alga 18.4ω6 & 11-OH-C18.3ω6 syn.	[27]	
Hamberg	1993	18:3ω3, 18.3ω6	8-^{2}H, 11-^{2}H	Alga 18.3ω6/ω3 oxygenation	[28]	
Hamberg & Gerwick	1993	10-HPOTrE (ω6)	8-^{2}H, 11-^{2}H	Alga fatty acid hydroperoxide isomerase	[29]	
Oliw et al.	1993	18:2ω6	11-^{2}H	HODE syn., rat liver microsomes	[30]	
Hamberg et al.	1994	18:2ω6	7-^{2}H, 8-^{2}H	Linoleate 8-dioxygenase/ isomerase	[31]	
Rao et al.	1994	18:2ω6	11-^{2}H	"LOX" activity of myoglobin	[32]	
Hamberg	1997	18:2ω6	11-^{2}H	C18.2 oxygenation by myoglobin	[33]	
Boeglin et al.	1998	20:4ω6	10-^{3}H	Human epidermis 12R-LOX	[34]	
Hamberg	1998	18:2ω6	11-^{2}H	COX-2	[35]	
Hamberg et al.	1998	18:2ω6	11-^{2}H	Manganese LOX	[36]	
Hombeck et al.	1999	9S-HPETE	16-^{2}H	Diatom dictyopterene A synthesis	[37]	
Rickert & Klinman	1999	18:2ω6	11-^{2}H	Soybean LOX-1 [+ label synthesis]	[38]	
Schneider & Brash	2000	20:4ω6	13-^{3}H	Acetylated COX-2, 15R-HPETE syn.	[39]	
Schneider et al.	2000	20:4ω6	10-^{3}H, 13-^{3}H	Synthesis of label, LOX, COX	[40]	
Schneider et al.	2001	20:4ω6	10-^{3}H	12S-LOX, 12R-LOX	[41]	
Peng et al.	2002	20:4ω6	13-^{2}H	Synthesis of label	[42]	
Hamberg et al.	2002	18:3	2-^{2}H	α-Dioxygenase	[43]	
Coffa et al.	2005	18:2ω6	11-^{3}H	Soybean LOX-1 mutants	[44]	
Hamberg	2005	9S- & 13S-HPODE	8-^{2}H, 14-^{2}H	Plant divinyl ether synthase	[45]	
Garscha et al.	2007	18:2ω6, 8R-HPODE	5-^{2}H, 8-^{2}H, 11-^{2}H	*Aspergillus* dioxygenases	[46]	

Table 1 continued

Author(s)	Year	Fatty acid	Label	Reaction (Enzyme)	Reference	Reaction, or synthetic product
Andreou et al.	2010	18:2ω6	11-^{2}H	Cyanobacterial linoleate 11-LOX	[47]	
Jernerén et al.	2010	18:2ω6	11-^{2}H	*Aspergillus terreus* dioxygenase	[48]	
Jernerén et al.	2010	18:2ω6	5-^{2}H, 8-^{2}H, 11-^{2}H	*Aspergillus clavatus* dioxygenase	[49]	
Jernerén et al.	2010	18:2ω6	7-^{2}H, 8-^{2}H, 11-^{2}H	*Magnaporthe oryzae* dioxygenase	[50]	
Martinez et al.	2010	18:2ω6	7-^{2}H, 8-^{2}H	*P. aeruginosa* dioxygenase	[51]	
Hamberg	2010	18:2ω6	11-^{2}H	Singlet oxygen	[52]	

relationship of hydrogen abstraction and oxygen insertion (Fig. 1a) [2, 3, 9, 72].

The antarafacial relationship in dioxygenase reactions contrasts with the predominantly suprafacial character of cytochrome P450 monooxygenation first observed already in the late 1950s ([73]; for review, see Ref. [74, 75]) (Fig. 1b). In the case of cytochrome P450, the oxygen rebound mechanism is sufficiently fast to secure hydroxylation of the substrate mainly on the face suprafacial to the initial hydrogen abstraction. There is, however, sufficient time-lapse (on the order of picoseconds) to allow some rotation of substrate prior to oxygen rebound, thus giving partial racemization to the process, observed as less than "pure" suprafacial oxygenation (e.g. Refs. [30, 76, 77]).

The fundamentals of desaturase mechanism were also revealed using stereospecifically labeled substrates (included in the following reviews, Refs. [78–81]). Invariably the initial and rate-limiting hydrogen abstraction occurs on the carbon nearest the fatty acid carboxyl (or polar head-group, e.g. in ceramides, the amide linkage to sphingosine), followed by the second hydrogen removal, and depending on the conformation of the carbon chain, forming either a *cis* or *trans* double bond (Fig. 1c).

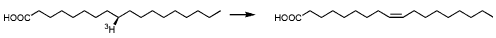
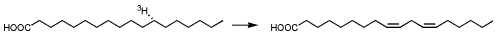
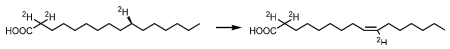
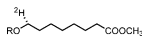
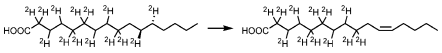
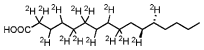
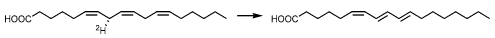
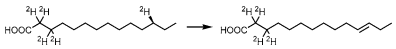
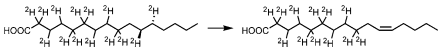
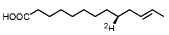
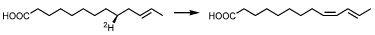
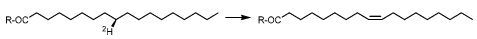
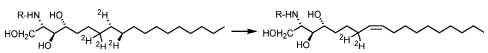
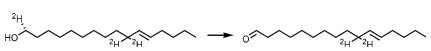
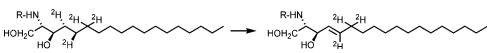
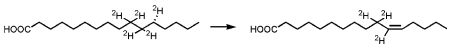
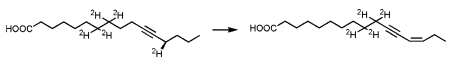
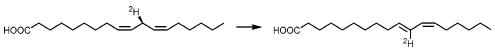
Applications related to desaturases are summarized in Table 2 and the order of the stereospecific hydrogen abstractions in Fig. 1c, and otherwise desaturase biochemistry is not discussed further in our review. For insightful analysis of the mechanism of desaturases as it relates to stereospecific hydrogen abstractions, we particularly recommend the excellent review by Buist [81], also Shanklin and Cahoon's review of desaturase biochemistry [82], and the earlier classic on stereospecific hydrogen abstractions by Morris [78].

Insights from Kinetic Isotope Effects

Experiments with stereospecifically-labeled substrates provide not only an understanding of the reaction stereochemistry but also further insights into the enzymatic mechanism. As outlined by Fersht [83], it is easier to break a C–H bond compared to a C–D bond by a factor of 7 at 25 °C. Because of the greater mass of tritium, C–T bonds are broken even more slowly (by a factor of $\sim$16.5 over C–H). These differences translate into kinetic isotope effects (KIE) in enzymatic reactions involving abstraction of a deuterium or tritium label. If abstraction is the rate-limiting step, as commonly occurs, then the heavy atom-labeled molecules will react at a slower rate, giving one measure of the KIE. Furthermore, in competitive reactions involving both labeled and unlabeled substrate molecules, during the course of the reaction there will be a gradual enrichment of the heavy atom-labeled molecules in the pool of unreacted substrate, providing a second parameter for quantitative analysis. Alternatively, if the H-abstraction is stereo-random and occurs after the commitment to reaction, the isotope effect can result in an enrichment of heavy atom label in the product: with the option of losing a hydrogen or deuterium/tritium from the same carbon, the KIE will lead to selective retention of the heavy atom (e.g. Ref. [18]).

Quantitative differences in reaction rate reflect the enzymatic mechanism involved in bond breaking. Purely

Table 2 Use of stereospecifically-labeled fatty acids in mechanistic studies on desaturases and isomerases

Author(s)	Year	Fatty acid	Label	Reaction (Enzyme)	Reference	Structure
Schroepfer & Bloch	1965	18:0	9-^{3}H, 10-^{3}H	Desaturation, *C. diphtheria*	[1]	
Morris et al.	1967	18:0	9-^{3}H, 12-^{3}H	Desaturation, *Chlorella*	[53]	
White	1980	16:0	10-^{2}H	*E. coli* desaturase	[54]	
Rawlings et al.	1989	18:0	[2-^{2}H]C9-diCOOH	*C. cladosporioides* Δ9-desaturase	[55]	
Boland et al.	1993	16:0	11-^{2}H, 12-^{2}H	moth Δ11-desaturase	[56]	
Frössl & Boland	1993	16:0	11-^{2}H, 12-^{2}H	Synthesis of label	[57]	
Wise et al.	1994	18:3ω6	8-^{2}H, 11-^{2}H	Alga, conjugated triene FA syn.	[58]	
Navarro et al.	1997	14:0	11-^{2}H, 12-^{2}H	*Spodoptera littoralis* desaturase	[59]	
Svatos et al.	1999	16:0	11-^{2}H, 12-^{2}H	Moth acyl-CoA Δ11-desaturase	[60]	
Abad et al.	2000	13:0	9-^{2}H, 10-^{2}H	Synthesis of label	[61]	
Abad et al.	2001	13:0	9-^{2}H, 10-^{2}H	Moth Δ9-desaturase of 11*E*−14:1	[62]	
Behrouzian et al.	2002	18:0	9-^{2}H	Castor stearoyl-ACP Δ9-desaturase	[63]	
Beckmann et al.	2002	16:0	6R-^{2}H, 7R-^{2}H	Sunflower Δ8-sphingolipid desaturase	[64]	
Hoskovec et al.	2002	16:1-ol	1-^{2}H	Moth fatty alcohol oxidation	[65]	
Beckmann et al.	2003	16:0	2R-^{2}H, 3*S*-^{2}H	*C. albicans* Δ4-sphingolipid desaturase	[66]	
Abad et al.	2004	16:0	12-^{2}H	Synthesis of label; moth desaturase	[67]	
Carlsson et al.	2004	18:1ω9	12-^{2}H, 13-^{2}H	Moss and dicot plant desaturase	[68]	
Abad et al.	2007	(11Z)-16:1	13-^{2}H, 14-^{2}H	Moth Δ13-desaturase	[69]	
Tremblay et al.	2007	16:0	4-^{2}H	*H. helix*, Δ4 dehydrogenation	[70]	
Liavonchanka et al.	2009	18:2ω6	11-^{2}H	*Propionibact. acnes* FA isomerase	[71]	

based on bond energies, one might expect a relative rate for k_H/k_D of ~7, yet distinctly different numbers are observed with specific enzymes. The exceedingly high deuterium KIE of ~80 associated with hydrogen abstraction in LOX catalysis is used to infer a lower energy transition pathway available only for the H-labeled species, a phenomenon known as hydrogen tunneling [38]. By contrast, a recent estimate for the deuterium KIE in COX catalysis is only 1.6–2.3 [84], clear evidence for the different mechanisms employed by LOX and COX for hydrogen removal. Those mechanisms center around a proton-coupled electron transfer to non-heme iron in LOX enzymes [85, 86], compared to abstraction of a hydrogen atom by a tyrosyl radical in COX [87, 88]. Further distinctions are observed

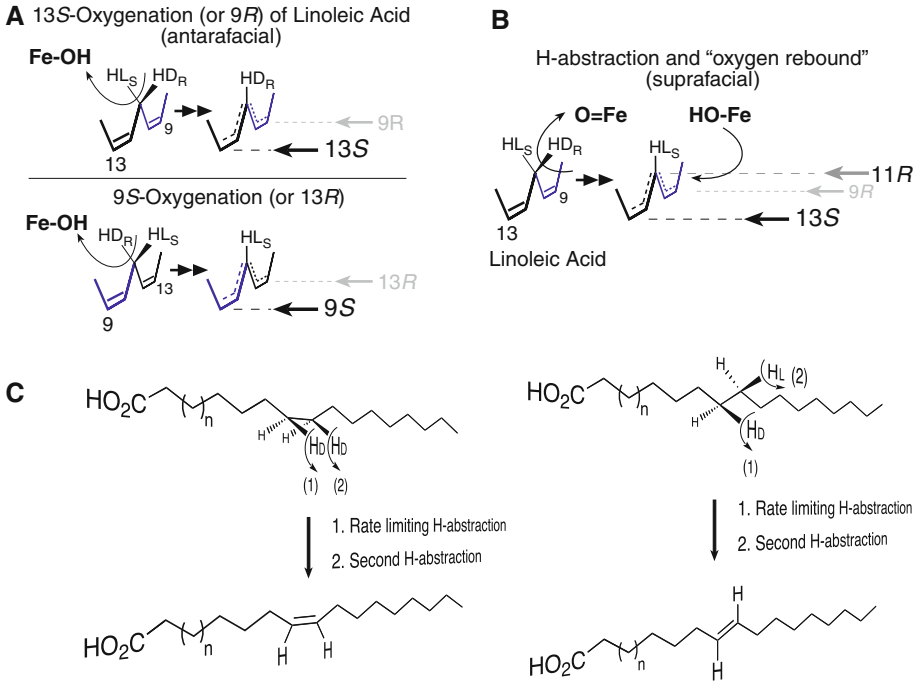


Fig. 1 Stereospecific hydrogen abstractions in fatty acid biochemistry. **a** The antarafacial relationship of the initial hydrogen abstraction and oxygen insertion, a characteristic feature of cyclooxygenase and lipoxygenase catalysis, here illustrated on linoleic acid in either of two head-to-tail orientations in the active site. **b** Suprafacial hydrogen abstraction and oxygenation as typified in cytochrome P450 catalysis, and illustrated here on linoleic acid in one of the two possible head-to-tail orientations. **c** Stereospecific hydrogen abstractions associated with desaturation to produce a *cis* double bond (*left side*), or *trans* (*right side*)

in association with hydrogen abstraction by cytochromes P450, which exhibit deuterium KIE values more reflective of relative bond energies, ~9 in aliphatic hydroxylations [89]; these P450 data are interpreted as consistent with the abstraction–recombination mechanism for aliphatic hydroxylation [90].

Stereochemistry of the Hydrogens in Fatty Acids

Many of the older papers assign chirality and prochiral hydrogens using the Fischer (D/L) system, which is less commonly used nowadays, although it does have an advantage in some situations. Because the Fischer system is based solely on the left or right orientation of the hydrogen in space, changing the nearby substituents has no effect on the D/L designation, whereas it might or might not change the *R*/*S* assignment based on Cahn-Ingold-Prelog. For substituents on fatty acids, making the D or L assignment is easy. Imagine the molecule hanging down with the carboxyl group on top. The chiral or prochiral carbon is then pointed towards the viewer (as in Fig. 2) and from there the hydrogen, hydroxyl, or other substituent on the chain is either on the right side (D), or the left (L).

A couple of specific examples illustrate the useful features of the Fischer nomenclature. The prochiral 11L-hydrogen in linoleic acid is also 11L in γ-linolenate or α-linolenic acid because it occupies the same position in space (on the left, when observed as in Fig. 2). However, adding the 15,16 double bond in α-linolenate changes the priority order at C-11 such that 11L is pro-*S* in C18:2ω6 or C18:3ω6, yet pro-*R* in C18:3ω3. For the same reason, the pro-*R* and pro-*S* hydrogens at C-10 in arachidonate (designated using both systems as D_R and L_S, respectively), remain as D or L in 15-hydroperoxy-eicosatetraenoic acid (15-HPETE), but the pro-*R*, pro-*S* designations reverse. To get around this potentially confusing issue with the *R*/*S* system, sometimes there is a cautionary note to the

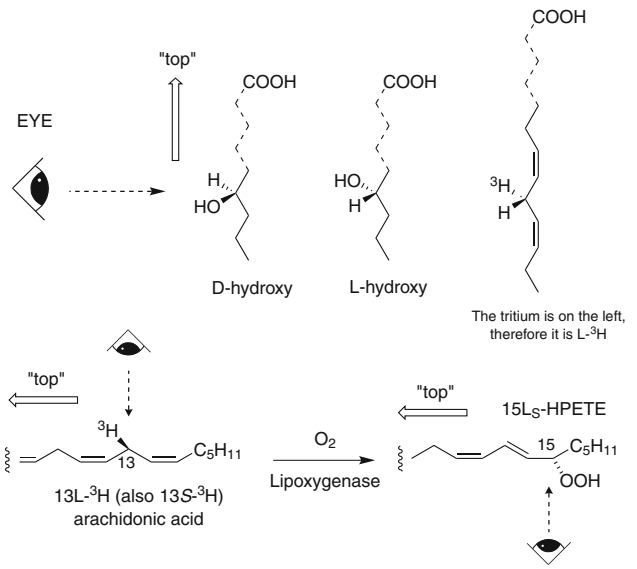


Fig. 2 Prochiral hydrogens in fatty acids, D/L assignment. With the fatty acid carboxyl on top and the carbon bearing the substituent aligned towards the viewer, the assignment is D (on the *right*) or L (*left*). The figure is a slight modification of an earlier version, reproduced with permission from Academic Press [137]

effect that chirality assignments throughout a report refer to the assignment in the parent fatty acid.

Synthesis of Stereospecifically Labeled Fatty Acids

Numerous fatty acids stereospecifically labeled with deuterium or tritium have been prepared by total organic synthesis, starting either with chiral natural products or by sequences involving chemical or enzymatic resolution of intermediates [17, 37, 42, 62, 64–67, 69, 70]. For example, a potentially general approach involving resolution of terminal epoxides was reported for synthesis of both enantiomers of palmitic acid stereospecifically deuterated at C-4 or C-5 [70]; the method offers flexibility and generality for synthesis of various saturated deuterated acyl chains simply by selection of the desired chain lengths of the building blocks. An interesting extension of such syntheses is to generate an isotopically labeled non-chiral compound into which chiral center(s) are introduced by an enzymatic method. Thus, [2*R*,3*R*-$^{2}H_{2}$]heptanoic acid was generated by exposing [2,3-$^{2}H_{2}$]2*E*-heptenoic acid to the enoate reductase of *Clostridium tyrobutyricum* [56, 60]. Alternatively, the unlabeled 2*E*-heptenoic acid was incubated with the reductase in $^{2}H_{2}O$ to afford [2*S*,3*S*-$^{2}H_{2}$]heptanoic acid. In another study, soybean lipoxygenase-1 was used to prepare [11*S*-^{2}H]linoleic acid from synthetically prepared [11*RS*-$^{2}H_{1}$]linoleic acid (i.e. singly deuterated linoleate, racemic at C-11), a process which is based upon the strong kinetic deuterium isotope effect accompanying the lipoxygenase-catalyzed conversion. As a consequence of this isotope effect, molecules containing a C-11 pro-*S* deuterium are almost unreactive and accumulate as [11*S*-$^{2}H_{1}$]linoleic acid, while the molecules unlabeled in the pro-*S* position (which are deuterated at pro-*R*) are removed by enzymatic transformation to hydroperoxide [38, 52].

A common strategy for stereospecific introduction of isotope into acyl chains, used already in the first study in this area [1], is based on the availability of enantiomerically pure long-chain hydroxy derivatives, most often hydroxystearates, as starting materials. These are converted into the corresponding *p*-toluenesulfonate derivatives which are treated with lithium aluminum deuteride/tritiide for introduction of isotope. It has been clearly shown that substitution of the *p*-toluenesulfonyloxy group by deuteride takes place as an S_N2 reaction resulting in inversion of the absolute configuration of the carbon attacked [91]. The required chiral hydroxy fatty acids can in many cases be isolated in optically pure form from natural sources; examples are ricinoleic acid, *β*-dimorphecolic acid and 2*R*-hydroxylinolenic acid (for review, see Ref. [92]). Furthermore, certain optically active hydroxy acids such as 9*R*- and 13*S*-hydroxyoctadecanoates can easily be prepared by catalytic hydrogenation of lipoxygenase-generated fatty acid hydroperoxides. Stereospecifically labeled stearates may also be prepared by chemical synthesis [4, 14, 18, 45]. This may involve an optically active short-chain hydroxy acid as starting material, obtained from commercial sources, as a natural product or prepared by resolution of an alkaloid salt of the racemic compound. Additionally, enantiomerically pure 3*R*-hydroxy acids can be obtained from polyhydroxyalkanoates [93] or prepared by enzymatic reduction of the corresponding *β*-keto acid using baker's yeast [46]. Subsequent elongation to the required chain-length can conveniently be performed using anodic coupling (the so-called Kolbe synthesis; see Ref. [94]). Chiral HPLC separation of racemic *p*-toluenesulfonate derivatives of hydroxystearates labeled with tritium at the chiral carbon followed by elimination of the tosylate by $LiAlH_4$ reduction offers an additional possibility to generate stereospecifically labeled stearates [40].

In the vast majority of papers given in Table 1, the above-mentioned procedures end up with stereospecifically labeled stearic acids. For studies of dioxygenases, which most often operate on mono- or polyunsaturated fatty acids of 18 or 20 carbon chain lengths, the labeled stearates have to be desaturated. Four organisms have found use for such conversions, *i.e. Tetrahymena pyriformis*, *Saprolegnia parasitica*, *Chlorella vulgaris*, and a readily available mutant of *Saccharomyces cerevisiae*. *Tetrahymena* is a ciliated protozoan which efficiently takes up exogenous stearate converting it mainly to *γ*-linolenate without noticeable scrambling of the isotope label [4]. Also labeled oleate and linoleate can be prepared using this organism. The proportion of unsaturated fatty acids in *Tetrahymena* is temperature-dependent; lower temperatures (20–25 °C) favor formation of *γ*-linolenate. *Saprolegnia parasitica* converts added stearate mainly to linoleate, arachidonate, and 5,8,11,14,17-eicosapentaenoate [95]; a reversed-phase HPLC chromatogram of the profile of fatty acids is illustrated in Schneider et al. [40]. The green alga *Chlorella vulgaris* has been used to prepare linoleate from stearate [10]. A practical disadvantage associated with these biodesaturations is dilution of the specific activity of the labeled material with endogenous unlabeled fatty acid. The extent of dilution can be severe, perhaps in the order of ten- to 100-fold, and particularly confounds the analysis of protium/deuterium ratios by mass spectrometry. A mutant of *S. cerevisiae* constructed by Reed et al. [96] converts labeled stearates to oleates and linoleates with very little isotopic dilution. This methodology, which was used by the original authors to prepare [12-^{2}H]-, [13-^{2}H]- and [18,18,18-$^{2}H_{3}$]-linoleic acids, is readily adaptable for production of stereospecifically labeled oleates and linoleates.

A new approach to the generation of stereospecifically-labeled unsaturated fatty acids involves chemical rearrangements of a specific fatty acid hydroperoxide via a chiral epoxyalcohol, with dehydroxylation-desaturation leading to a primary alcohol that is oxidized back to the original (now stereospecifically ^{2}H-labeled) polyunsaturated fatty acid (Fig. 3). Potentially the method is generally applicable to any polyunsaturated fatty acid, if sufficient chiral fatty acid hydroperoxide is available. A very active lipoxygenase such as soybean LOX-1 can be used to prepare 100+ milligrams of ω6 hydroperoxides as starting material, and the use of other natural or recombinant enzymes could broaden the scope of this method.

Lipoxygenase Mechanism, Stereospecific Hydrogen Abstraction, in Historical Context

The stereochemical features of lipoxygenase-catalyzed oxygenation as we understand them today were first described by Hamberg and Samuelsson [2, 97]. They established both the stereochemistry of oxygenation of

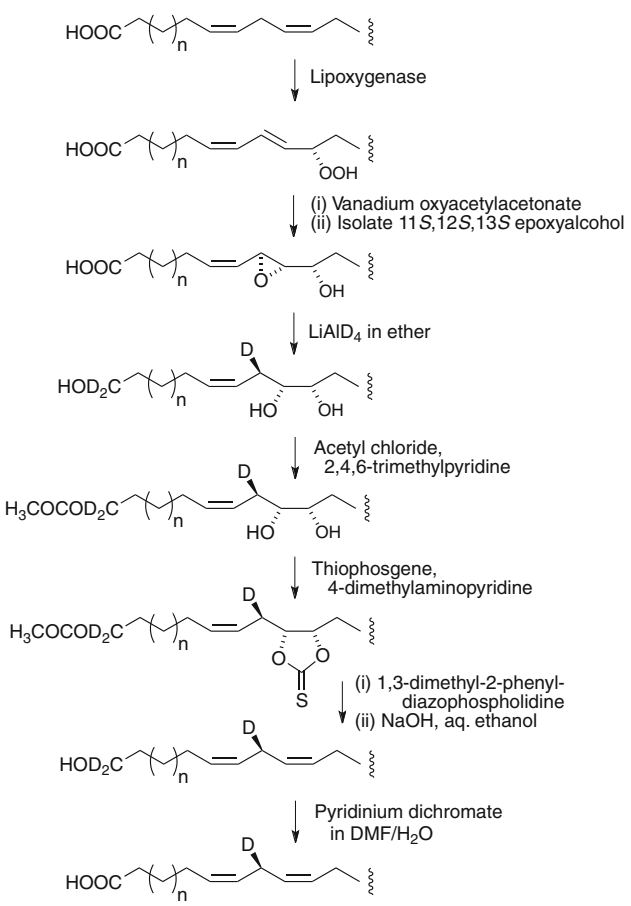


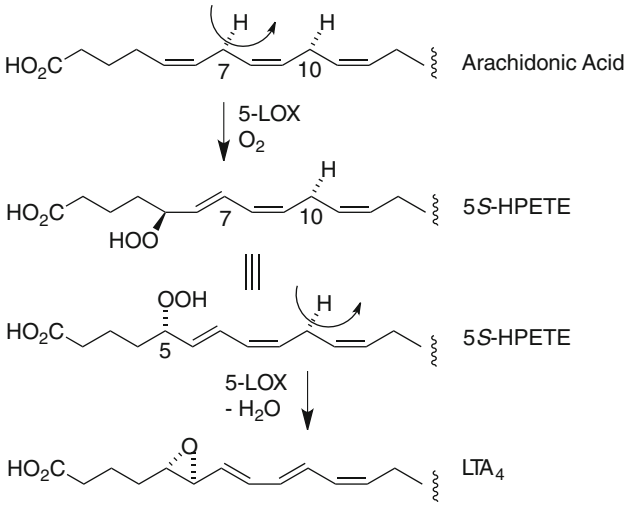
Fig. 3 Synthesis of stereospecifically labeled polyunsaturated fatty acid via a chiral fatty acid hydroperoxide and epoxyalcohol [52]

polyunsaturated fatty acids and the stereospecificity of the associated hydrogen abstraction. Prior to their reports, there was some indication that lipoxygenase reactions differ from autoxidation in producing chiral product(s) with optical rotation [98], but the precise structure(s) were unknown. Hamberg and Samuelsson showed that the soybean lipoxygenase oxygenates C18 and C20 polyunsaturated substrates mainly in the ω6 position, i.e. producing the 13-hydroperoxide product of linoleic or linolenic acids and the 15-hydroperoxide of the C20 substrates [97]. The *S* stereochemistry of these products was established, both by hydrogenation of 13-hydroxylinoleate and comparison of its optical rotation with known derivatives such as ricinoleic acid, and by oxidative ozonolysis and optical rotation of the resulting α-hydroxy-heptanoate fragment [97]. Using stereospecifically labeled [13D-^{3}H]- and [13L-^{3}H]20:3ω6, the specific removal of the 13L (pro-*S*) hydrogen was demonstrated in forming the 15L$_S$-hydroperoxide. A strong kinetic isotope effect associated with this transformation proved that H-abstraction is, or coincides with, the rate-limiting step of lipoxygenase catalysis [2].

Further analysis of lipoxygenase stereochemistry over the next twenty years established the existence of other *S*-specific lipoxygenases, which in all cases were found to operate with an antarafacial relationship of hydrogen abstraction and oxygenation. These enzymes included the plant 9*S*-LOX [9, 99], and the mammalian leukocyte 5*S*-LOX [17, 100], platelet 12*S*-LOX [12, 101, 102], and reticulocyte and leukocyte 15*S*-LOX (and its rodent and porcine homologue 12/15-LOX) [24, 103, 104].

In plants, linoleic acid is commonly oxygenated by either 9*S*-LOX or 13*S*-LOX. Both exhibit the antarafacial relationship, but the reactions are initiated on opposite faces of the substrate and their product hydroperoxides are also on opposite sides. Indeed, the results of experiments with stereospecifically-labeled linoleic acids indicated that 9*S*-oxygenation is associated with removal of the 11D hydrogen whereas 13*S* oxygenation is associated with removal of the 11L hydrogen [2, 9]. One explanation is that the oxygenation machinery in the enzyme active site of these two types of lipoxygenase are spatially reversed, although this is highly unlikely in view of their close structural homology [105]. Far more credibly, the linoleate aligns in opposite head-to-tail orientation in 9*S*-LOX and 13*S*-LOX, thus presenting either the 11D or 11L hydrogen for abstraction (Fig. 2a), a concept that was deduced by Egmond, Vliegenthart and Boldingh who first made the observation on the opposite hydrogen abstractions [9].

In the early 1980s, the recent discovery of the leukotrienes prompted many studies on their cellular biosynthesis, and the search was on for the leukotriene A (LTA) synthase that catalyzes the committed step into the LT



Scheme 1

pathway. The discovery that the LTA synthase is none other than the lipoxygenase that produces the HPETE substrate was quite unexpected at the time. It was surprising because there was no such precedent from analysis of the soybean lipoxygenase, upon which almost all LOX biochemistry was founded. Initially, one of the main lines of evidence making the connection between LOX and LTA synthase—even before purification of the enzyme—was the demonstration that LTA epoxide synthesis proceeds with an initial stereospecific hydrogen abstraction, the well-established feature of lipoxygenase catalysis [13–17]. Taking leukocyte 5-LOX as the prototypical example, in forming 5*S*-HPETE the enzyme catalyzes a stereospecific hydrogen abstraction from C-7, then in a simple "frame-shift" along the carbon chain to the next available pentadiene, hydrogen abstraction from C-10 initiates the synthesis of LTA_4 (Scheme 1). As suggested in Scheme 1, to form LTA_4 as the correct *trans* epoxide requires positioning of the hydroperoxide such that it is suprafacial to the C-10 hydrogen abstraction, as the lipoxygenase iron participates in both the H-abstraction and cleavage of the hydroperoxide (cf. Refs. [106–108]). Ultimately several lipoxygenases were purified and shown to catalyze the conversion of HPETE to an LTA-type epoxide [21, 104, 109, 110].

The apparent occurrence of exclusively *S* lipoxygenases in Nature was first contradicted by the report of Gordon Bundy and colleagues of the Upjohn Company that arachidonic acid is converted to its 8*R*-hydroperoxide (8*R*-HPETE) by extracts of the Caribbean coral *Plexaura porosa* [111]. It was soon found that other corals and marine invertebrates also produce 8*R*-HPETE or other *R*-configuration hydroperoxides (e.g. Refs. [23, 112–117]), although at first there was no indication whether this new type of enzyme was structurally related to the lipoxygenases of plants and higher animals. That 12*R*-HPETE is produced in sea urchin eggs via an antarafacial relationship of H-abstraction and oxygenation was reported by Hawkins and Brash [118], and later the same relationship was shown for 8*R*-HPETE in coral extracts by Hughes and Brash [26]. A decade later, a search for the enzyme responsible for biosynthesis of 12*R*-HETE in human skin led to the cloning and expression of mammalian 12*R*-lipoxygenase; the report demonstrated the now universally observed antarafacial relationship of H-abstraction and oxygenation in lipoxygenase catalysis, in this case the formation of 12*R*-H(P)ETE by recombinant human 12*R*-LOX and in samples of human skin containing the enzyme activity [34].

Purification and molecular cloning of an enzyme producing 8*R*-HPETE from the coral *Plexaura homomalla* proved that the 8*R*-lipoxygenase belongs to the same enzyme superfamily as the *S*-LOX already characterized [119]. More recent X-ray crystallographic analysis of a *P. homomalla* 8*R*-LOX [120] demonstrated that the coral 8*R*-LOX protein has a very similar overall topology to the plant and mammalian *S*-specific LOX [121–123]. Meanwhile, a search for active site residues that might influence *R* or *S* oxygenation specificity in lipoxygenases resulted in the discovery of a critical residue, conserved as Gly in *R*-LOX and Ala in *S*-LOX [124]. This provided evidence that rationalized the difference between *R* and *S* LOX enzymes. Oxygenation can occur at either end of the reacting pentadiene, at one end giving the *S* configuration, at the other *R* (Fig. 1a). Switching the Ala-to-Gly residue or vice versa by site directed mutagenesis induces this transformation between *R* and *S* oxygenation. Following up on this finding, it was shown using stereospecifically 11-^{3}H-labeled linoleic acids and soybean lipoxygenase with mutated active site Ala-to-Gly that, as had been predicted, 9*R* and 13*S* oxygenation of linoleic acid involve the same initial hydrogen abstraction with oxygenation occurring at opposite ends of the same face of the reacting pentadiene (Fig. 1a) [44]. Similarly, the equivalent Ala-to-Gly mutation in 9*S*-LOX induces the formation of 13*R*-product [125].

The effects of this Ala-to-Gly interconversion are now substantiated with several other LOX enzymes [126]. Occasionally there is a twist to the "rule" such as the natural occurrence of the larger Ala in association with *R*-LOX activity, with site-directed mutagenesis to Val or Ile inducing formation of *S*-configuration products [127, 128]. Interestingly, there is a naturally occurring Ala-to-Gly "mutant" lipoxygenase in the olive plant (GenBank accession no. EU678670) encoding Gly568 rather than the expected Ala. In accord with the above concepts, the enzyme produces 13*R*-HPODE in addition to 9*S*-HPODE [129], as predicted from the results of site-directed mutagenesis experiments with recombinant LOX enzymes.

Cyclooxygenase Mechanism, Stereospecific Hydrogen Abstraction, in Historical Context

Fundamental insights into the cyclooxygenase reaction of prostaglandin biosynthesis were reported by Hamberg and Samuelsson in two papers published side-by-side with their soybean lipoxygenase studies [2, 4]. At the time (1967) it was recognized that prostaglandins are formed from a linear C20 polyunsaturated fatty acid via a putative endoperoxide intermediate in which a cyclopentane ring is comprised of carbons 8–12, with the two atoms of a molecule of molecular oxygen bridging C-9 and C-11, and another oxygen molecule reacting at C-15. Using two labeled eicosatrienoic acids (20:3ω6) with stereospecific tritium at the 13-carbon, the authors demonstrated that the 13L$_S$ hydrogen is selectively removed in the transformation of 20:3ω6 to prostaglandin E$_1$ [4]. A strong associated kinetic isotope effect, which resulted in the gradual enrichment of the specific activity of the unreacted substrate during the course of the transformation, indicated that 13L$_S$ hydrogen abstraction is, or coincides with, the initial and rate-limiting step of prostaglandin biosynthesis. This alone provided circumstantial evidence that the initial oxygenation of the fatty acid occurs at C-11 and that the resulting 11-peroxyl radical goes on to form the peroxide bridge spanning C-11 to C-9 in the endoperoxide intermediate.

Additional studies on the formation of cyclooxygenase side products also made use of the labeled [13-^{3}H]20:3ω6 substrates [2]. The 12-hydroxy-C$_{17:2}$ fatty acid side-product (the 20:3 equivalent of 12-hydroxyheptadecatrienoic acid (HHT) from arachidonic acid) was shown to exhibit a similar stereospecific loss of the 13L$_S$ hydrogen and retention of the 13D$_R$ hydrogen as occurs in biosynthesis of PGE$_1$. Use of other 20:3ω6 substrates with tritium labels at either C-9, C-10, C-11 or C-15, provided the proof that this 12-hydroxy-C$_{17:2}$ side product is formed with loss of three carbons from the cyclopentane ring. Furthermore it was noted that cyclooxygenase substrates that gave rise to the C$_{17}$-OH by-product also formed significant thiobarbituric acid reactive substances (indicative of an aldehyde), and indeed, the radioactive 3-carbon fragment formed from [11-^{3}H]20:3ω6 was isolated and identified as malondialdehyde [2]. This line of evidence provided additional support for the formation of a cyclic endoperoxide as an intermediate in the formation of the stable prostaglandins.

In these early papers it was noted that C$_{18}$ polyunsaturated fatty acids are also metabolized by the preparation of COX-1 (sheep seminal vesicle microsomes) and the products from linoleic acid were identified as the 9- and 13-hydroxy derivatives [3]. In 1980, Hamberg and Samuelsson reported on the stereochemistry associated with transformation of [11-^{3}H]linoleic acid [11]. The 9-hydroxy product was mainly of the 9L$_R$ configuration (equivalent to the 11*R*-HETE by-product from arachidonic acid) and the other was mainly 13L$_S$-hydroxy. These were each formed with ~80% removal of the 11L (pro-*S*) hydrogen from the substrate. The stereo fidelity was not so high as found using the 20:3ω6 substrate, apparently reflecting less than perfect control of oxygenation of linoleic acid by COX-1. Later studies by Hamberg gave similar results using COX-2 [35]. The subsequent description of COX-1 crystal structures with bound substrate indicated that, similar to 20:3ω6 and arachidonic acid, linoleic acid lies in the cyclooxygenase active site with the appropriate bis-allylic hydrogen (the 13L$_S$ of the C20 substrates and 11L$_S$ of linoleic acid) poised for reaction with an incipient radical on Tyr-385 [130, 131]. By contrast to the better COX substrates, oxygenation of linoleic acid (and EPA) were much more severely compromised by active site mutations that modified the positioning of the α or ω ends of the fatty acid carbon chain [131]. Linoleic acid in particular has to assume a more stretched out α chain in order for the carboxyl to make contact and be stabilized by interaction with the Arg-120 and Tyr-355 at the entrance to the oxygenase substrate-binding channel, making the interactions more easily disturbed by mutagenesis within the COX active site.

In the early 1990s an interesting an unexpected arachidonic acid metabolite was detected upon reaction with aspirin-acetylated COX-2. Whereas aspirin inhibition completely blocks all oxygenase activity in COX-1, aspirin treatment of COX-2 blocks prostaglandin biosynthesis, but switches the oxygenation specificity to produce 15*R*-HETE as the only enzymatic product [132–134]. This 15*R* specificity is opposite to the usual 15*S* oxygenation associated with the final steps of prostaglandin endoperoxide biosynthesis. Accordingly, Schneider and Brash questioned the specificity of the hydrogen abstraction associated this 15*R*-HETE production. The result that might have been anticipated, based on parallels to *R* and S oxygenation in lipoxygenase catalysis, is that the substrate would assume a reversed orientation and that the H-abstraction would also be the reverse of normal. Using 13*R*-^{3}H and 13*S*-^{3}H arachidonic acids, however, it was found that the specificity matches that in normal prostaglandin production, i.e. specifically the pro-*S* hydrogen at C-13 is removed in formation of PG endoperoxide with normal COX-2 and in 15*R*-HETE synthesis in the aspirin-treated enzyme. This formation of 15*R*-HETE appears, therefore, to be the one known example in which the relationship of stereospecific hydrogen abstraction to oxygenation is suprafacial, rather than antarafacial. The authors rationalized this result by proposing that the main body of the substrate (including the 13-carbon) lies in the usual orientation, but the tail carbons are twisted over to expose the 15*R* position to attack by O$_2$ (Fig. 4). In discussion, it was further proposed that the

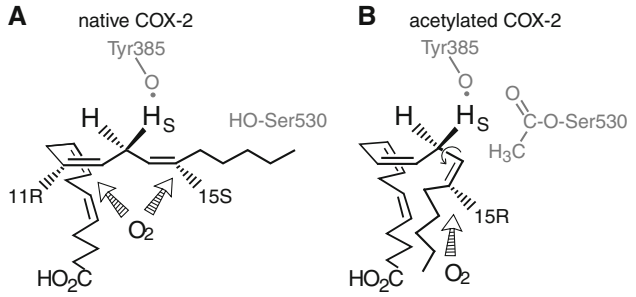


Fig. 4 Synthesis of 15*R*-HETE by aspirin-acetylated COX-2. Unlike all specific oxygenations by COX and LOX enzymes, this reaction exhibits suprafacial relationship of hydrogen abstraction and the 15*R* oxygenation. The figure is reproduced from Ref. [39] with permission

terms suprafacial and antarafacial begin to lose their utility under these circumstances [39].

Fatty Acid Monooxygenation and Stereospecific Hydrogen Abstraction

Cytochrome P450 is the prototypical fatty acid monooxygenase (hydroxylase and epoxygenase). The principles related to hydrogen abstraction in P450 reactions were mainly worked out using other substrates, and the "rules" subsequently shown to apply to fatty acid monooxygenation (reviewed in Refs. [74, 75] and in earlier editions of the book). Typically, the relationship of the hydrogen abstraction and oxygenation ("oxygen rebound") in P450 catalysis is suprafacial (Figs. 1b, 5). Unlike in LOX and COX reactions the activated oxygen in P450s is covalently bound to the reactive heme iron. Also, it is this very same ferryl species which catalyzes the H-abstraction. Depending on the substrate, there may be sufficient mobility within the active site so that it can spin around prior to oxygen rebound, in which case the relationship between H-abstraction and oxygenation appears to be antarafacial.

Therefore for the reasons cited, with many substrates there is an element of apparently stereorandom oxygenation following hydrogen abstraction in P450 catalysis. There may be some evidence of this in the results of linoleic acid oxygenation as reported by Oliw and co-workers in rat liver microsomes [30], yet mainly the results hold to the suprafacial relationship of H-abstraction and oxygenation. The major products detected were 9*R*-HODE (formed with 95% retention of deuterium from [11L$_S$-^{2}H]linoleic acid and 22% retention from [11D$_R$-^{2}H]linoleic acid), and 13*R*-HODE (24% retention from [11L$_S$-^{2}H]linoleic acid and 71% retention from the 11D$_R$-^{2}H). What may not have been discussed before is the concept that formation of these two products and their associated hydrogen abstractions can be accounted for by a change in the head-to-tail orientation of the substrate in the P450 active site (Fig. 5). This is akin to the concepts of reversal of substrate binding orientation that is well established in the lipoxygenase literature.

Formation of the minor hydroxy products in the same study (9*S*-HODE and 13*S*-HODE) can be rationalized similarly. The 9*S* position on linoleate is on the same face of the substrate as 13*R*, and by-and-large the results of Oliw et al show qualitatively similar deuterium retentions in the 9*S*-HODE and 13*R*-HODE products [30]. Based on these results, it can be inferred that the substrate is mainly in the same head-to-tail orientation in forming 9*S*- and 13*R*-HODE. A similar match applies for the major 9*R*-HODE and the minor 13*S*-HODE, which assume the

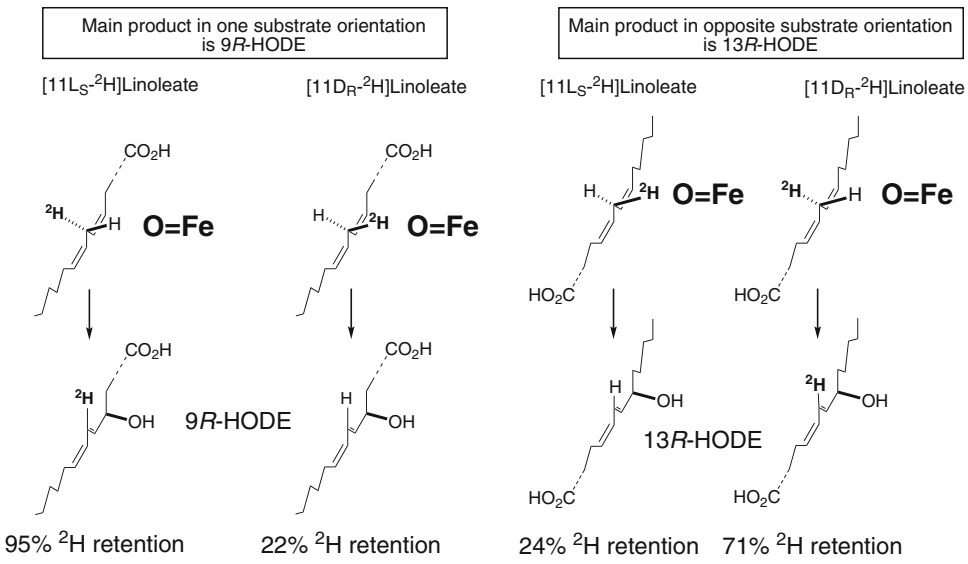


Fig. 5 Suprafacial H-abstraction and oxygenation in cytochrome P450 mediated oxidation of linoleic acid in rat liver microsomes [30]. The reported retentions of deuterium in the major products from [11D$_R$-^{2}H]- and [11L$_S$-^{2}H]linoleic acids are rationalized here in terms of two possible head-to-tail orientations of the substrate, thus accounting for formation of 9*R*-HODE and 13*R*-HODE with predominantly suprafacial hydrogen abstraction and oxygenation. A similar analysis can rationalize formation of the two minor products reported, 9*S*-HODE and 13*S*-HODE (not shown)

opposite orientation. It also follows from this line of thinking that each major product is formed by oxygenation at the "top" end of the pentadiene as cartooned in Fig. 5, while the minor products are each formed at the lower end.

Finally, the other significant mono-hydroxylated product in this study is 11-HODE, which showed 38% retention of the $11D_R$-^2H label and 69% retention of the $11L_S$-^2H. Because the $11D_R$ deuterium label is largely retained, and assuming a suprafacial relationship of H-abstraction and oxygenation, these data suggest that 11-HODE is mainly formed in the head-to-tail orientation on the right side of Fig. 5.

Future Prospects/Open Issues

The fundamentals of the catalytic mechanisms of lipoxygenases, cyclooxygenases, cytochromes P450, desaturases, and isomerases have long been established, yet new enzymes and reactions continue to arise. Quite recently, stereospecifically labeled fatty acids were applied in exploring the reaction mechanism of novel dioxygenases in fungal pathogens of the *Aspergillus* species and a similar type of enzyme in the bacterium *Pseudomonas aeruginosa* [48–51]; these enzymes have both dioxygenases and peroxidase (P450-related) domains which this mechanistic approach can help unravel. The discovery of a catalase-related hemoprotein from *Anabaena* PCC 7120 that converts 9*R*-hydroperoxylinolenic acid into a leukotriene A-type epoxide together with a highly unusual bicyclobutane fatty acid presents another opportunity to glean insights using this methodology [135]; the proposed intermediates in the transformation are carbocations, and an enzyme-catalyzed hydrogen abstraction is required in the course of the transformation. Equivalent reactions initiated on linoleic acid produce a conjugated diene analog of LTA [136]. Among other issues, comparison with the mechanism of the lipoxygenase-catalyzed route to LTA epoxides should be of interest.

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