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Opposite Stereochemical Courses for Enzyme-Mediated Alkene Reductions of an Enantiomeric Substrate Pair

Despina J. Bougioukou and Jon D. Stewart*

127 Chemistry Research Building, Department of Chemistry, University of Florida, Gainesville, Florida 32611

Received January 15, 2008; E-mail: jds2@chem.ufl.edu

Abstract: Rat NADP-dependent leukotriene B_4 12-hydroxydehydrogenase (Ltb4dh) catalyzes olefin reductions for some activated alkenes at the expense of NADPH in the absence of a flavin cofactor. Unlike flavoprotein alkene reductases, where net *trans*-addition of hydrogen has been consistently observed, Ltb4dh reduced both enantiomers of perillaldehyde to the same *cis*-product. To uncover the reason for this unexpected result, the stereochemical courses of perillaldehyde reductions by Ltb4dh were determined by deuterium labeling followed by ^{2}H NMR analysis. These data showed unequivocally that Ltb4dh mediated net *trans*-addition of hydrogen to (*R*)-perillaldehyde but followed the opposite stereochemical course (net *syn*-addition) for (*S*)-perillaldehyde. To the best of our knowledge, such divergent stereochemical pathways for a single enzyme have not previously been reported.

Enzyme-mediated carbonyl reductions using libraries of cloned proteins have proven highly valuable in providing chiral intermediates with high optical purities.[1–3] More recently, these efforts have been extended to stereoselective reductions of activated alkenes.[4] Flavoprotein alkene reductases, particularly the old yellow enzyme from *Saccharomyces pastorianus*,[5] have been well-studied with respect to both structure[6] and mechanism,[7,8] and several synthetic applications of this and related enzymes have been reported.[9–14] Old yellow enzymes catalyze net *anti*-addition of H_2 to electron-deficient alkenes. Hydride is supplied by reduced flavin mononucleotide (FMN, formed at the expense of NADPH), and addition of a solvent-derived proton (in some cases directly from an amino acid side chain such as a Tyr) completes the reduction. In addition to this large group of flavoprotein alkene reductases, a handful of additional enzymes reduce activated alkenes with no additional cofactors beyond NAD(P)H.[15–21] While examining the substrate and stereoselectivity of one of these flavin-free alkene reductases—rat NADP-dependent leukotriene B_4 12-hydroxydehydrogenase (Ltb4dh)—we discovered its unprecedented ability to transfer the elements of H_2 in either a *syn*- or *anti*-fashion, depending on the substrate's absolute configuration. To the best of our knowledge, the ability of a single enzyme to mediate two opposing stereochemical courses in alkene reductions has never been observed previously.

Ltb4dh was originally isolated as an alcohol dehydrogenase[22] and was later shown to mediate activated alkene reductions.[23] The efficiency of the latter reaction is approximately 300-fold higher, although both activities appear to be physiologically relevant. Dick and Kensler showed that the enzyme follows an ordered kinetic mechanism, with NADPH binding first and $NADP^+$ dissociating last with transfer of the 4*R*-hydrogen of NADPH.[21]

The gene encoding rat Ltb4dh[24] was PCR-amplified and inserted by homologous recombination into an *Escherichia coli* expression plasmid with an *N*-terminal glutathione *S*-transferase affinity tag to facilitate isolation.[1] Both (*R*)- and (*S*)-perillaldehyde were tested as substrates for the purified fusion protein

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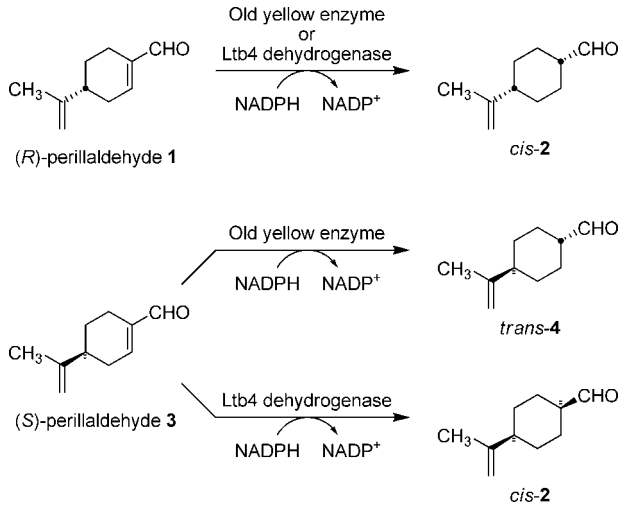
Scheme 1



(GST-Ltb4dh) in the presence of catalytic amounts of NADPH (supplied by enzymatic glucose-6-phosphate oxidation) (Scheme 1). For comparison, the same reactions were carried out with an analogous GST-fusion protein with *S. pastorianus* old yellow enzyme (GST-OYE). As expected,[7,12] the major products from both GST-OYE-mediated alkene reductions—*cis*-**2** from (*R*)-perillaldehyde and *trans*-**4** from (*S*)-perillaldehyde—reflected consistent net *anti*-addition of H_2, regardless of the substrate's absolute configuration. This was not the case for reactions catalyzed by GST-Ltb4dh, however: both perillaldehyde antipodes were reduced to the same product (*cis*-**2**).[25] To verify that a contaminating *E. coli* host protein was not responsible for these unexpected results, the GST affinity tag alone was expressed in *E. coli* and purified by the same method. Incubating an equivalent quantity of the GST affinity tag with either (*R*)- or (*S*)-perillaldehyde in the presence of NADPH (generated by glucose-6-phosphate dehydrogenase) gave no reaction, and the starting materials were recovered.

We considered three mechanisms to explain the unexpected formation of the same *cis*-product from both perillaldehyde antipodes by GST-Ltb4dh. One possibility was that the enzyme reduced (*S*)-**3** to *trans*-**4**, which then underwent subsequent nonenzymatic epimerization to yield only *cis*-**2**. This was not consistent with the behavior of *cis*-**2** and *trans*-**4** under our reaction conditions, which converted over time to a mixture heavily favoring *trans*-**4**. A second possibility was that both (*R*)- and (*S*)-perillaldehyde underwent net *anti*-addition of H_2 but bound to the active site with opposite alkene faces positioned for hydride addition. Finally, it was possible that hydride addition to both (*R*)- and (*S*)-perillaldehyde occurred on the same alkene face, but protonation took place on opposite sides. The latter two possibilities can be distinguished by their predicted stereochemical courses.

Because *cis*-**2** possesses a plane of symmetry, the relative configurations of both hydrogens added during the reduction had to be determined in order to establish the stereochemical pathways. Both (*R*)- and (*S*)-perillaldehyde were reduced by GST-Ltb4dh using a substoichiometric quantity of (*R*)-

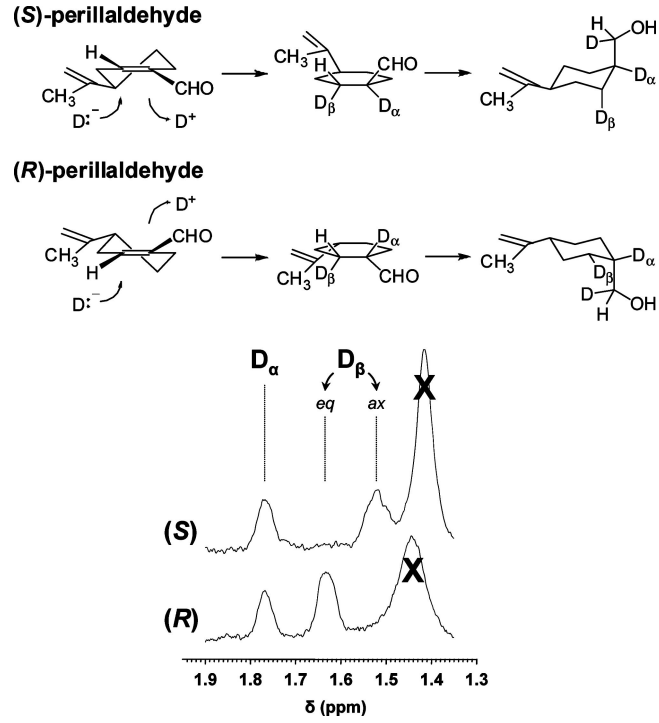


Figure 1. ^{2}H NMR spectra from perillaldehyde reductions carried out in the presence of NADPD and D_2O. Net *cis*-addition to (*S*)-perillaldehyde results in an axial β-deuterium, whereas net *trans*-addition to (*R*)-perillaldehyde affords an equatorial β-deuterium. The enzyme used to regenerate NADPD (*T. brockii* alcohol dehydrogenase) also catalyzed carbonyl reduction of *cis*-**2**, so that the primary alcohol was final product. The peaks marked by "X" are due to impurities.

NADPD in D_2O solvent. The reduced cofactor was prepared by *in situ* oxidation of *i*-PrOH-d_8 using *Thermoanaerobium brockii* alcohol dehydrogenase (TBADH), which also catalyzed carbonyl reduction of *cis*-**2**.[26] Since aldehyde reduction occurred *after* alkene reduction, however, it had no effect on the isotope configurations and simplified the product mixture by eliminating enolization that converted *cis*-**2** to *trans*-**4**.

Net *trans*-addition of D_2 to the conjugated alkene of (*R*)-perillaldehyde leads to equatorial deuterium atoms at both the α- and β-positions, and this was confirmed by ^{2}H NMR data (Figure 1). Net *trans*-addition of D_2 to the opposite face of (*S*)-perillaldehyde would also yield equatorial deuterium atoms at both the α- and β-positions. This was not observed, however (Figure 1). Instead, the deuterium at C_β was found only in the axial position, indicating that net D_2 addition had proceeded *via* a *cis*-pathway. These results clearly ruled out the possibility that the perillaldehyde enantiomers bound to the protein's active site with opposite alkene faces proximal to the nicotinamide ring. On the other hand, the data were consistent with the notion that opposite facial selectivity in the protonation phase was

(25) *Cis*-**2** was the major product of these conversions (>90%). A control reaction showed that *cis*-**2** isomerized non-enzymatically to a mixture heavily favoring *trans*-**4** under the reaction conditions, and this eroded the stereochemical purity of the product, particularly when long conversion times were required.

(26) Attempts to carry out these reactions using only GST-Ltb4dh and excess NADPD in D_2O were complicated by subsequent non-enzymatic epimerization of *cis*-**2** (see ref 25), which resulted in complex NMR spectra. See Supporting Information for details of these experiments. We did not detect (*R*)- or (*S*)-perillyl alcohol during these studies; (*R*)-perillyl alcohol could be detected only when (*R*)-**1** GST-Ltb4dh was ommitted from the reaction mixtures. Whether TBADH could reduce *trans*-**4** was not investigated since this product was not formed at significant levels during these experiments.

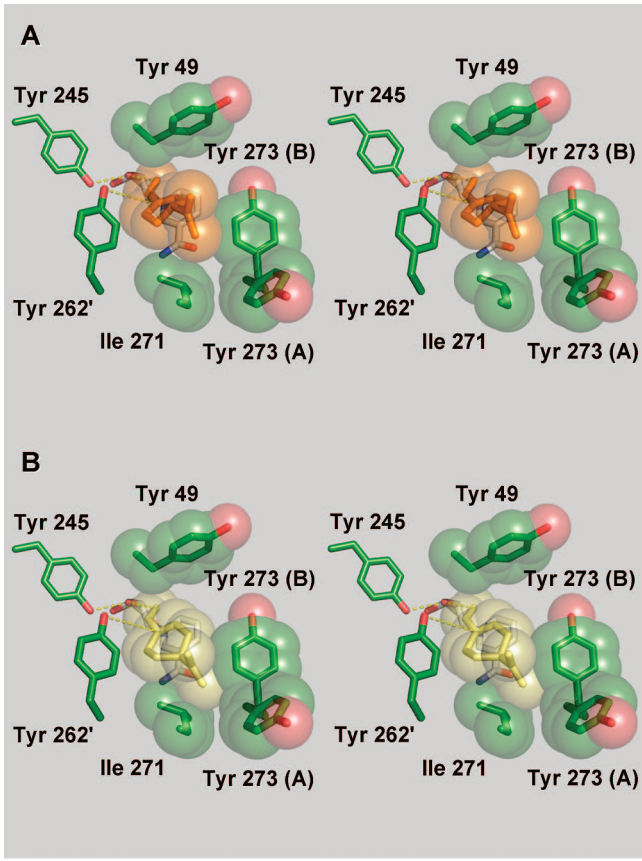


Figure 2. Modeled substrate binding modes for perillaldehyde enantiomers. (A) The enone moiety (C_α, C_β, and the carbonyl atoms) of (*R*)-perillaldehyde in its lowest-energy conformation (HyperChem 7.5) was overlaid with the corresponding atoms of 15-oxo-prostaglandin E_2 in its ternary complex with $NADP^+$ and guinea pig LTB4 dehydrogenase (PDB file 1V3V). Only residues in the vicinity of the substrate (orange) are depicted, along with their corresponding van der Waals spheres. The NMP portion of $NADP^+$ and the side chains of two tyrosine residues considered as potential proton donors (245 and 262, from the second subunit) are shown in stick form. The side chain of Tyr 273 was observed by Hori et al. in two alternate conformations (A and B),[28] and both are shown. (B) As in panel A, except that the enone moiety of (*S*)-perillaldehyde (yellow) was overlaid with the corresponding atoms of 15-oxo-prostaglandin E_2. Figures were rendered in PyMol.[37]

responsible for the different stereochemical courses of the reductions.[27]

No three-dimensional structure is currently available for rat Ltb4dh; however, Hori et al. have solved the X-ray crystal structure for the closely related guinea pig homologue with bound $NADP^+$ and a substrate (15-oxo-prostaglandin E_2).[28] We modeled potential Michaelis complexes of rat Ltb4dh with both (*R*)- and (*S*)-perillaldehyde in the presence of the bound nicotinamide cofactor. The (*R*)-enantiomer could be easily accommodated in the active site with its alkene stacked above the nicotinamide ring, consistent with the observed trajectory of hydride addition (Figure 2A). The opposite face of the alkene faced an open pocket likely filled with solvent during the normal catalytic cycle, an arrangement that would yield net *trans*-addition of H_2, as observed. By contrast, it was not possible to dock (*S*)-perillaldehyde without severe steric clash, particularly with the side chains of Ile 271 and Tyr 273 (Figure 2B). One possible response would be to tilt the cyclohexenyl ring up and away from the nicotinamide ring, which might allow the same face of the alkene to accept both a hydride and a proton.

The general acid(s) used by Ltb4dh to transfer a solvent-derived proton during enone reductions is unknown. In *S. pastorianus* old yellow enzyme, the side chain of Tyr 196 acts as a general acid, and Phe substitution at this position blocks enone reduction.[7] The side chain of Tyr 262 is positioned somewhat similarly in our computer model, suggesting a possible role in the net *trans*-addition of H_2 to substrates such as (*R*)-perillaldehyde. This proved not to be the case, however, since the corresponding Phe mutant (Y262F) was even more catalytically active against both (*R*)- and (*S*)-perillaldehyde. The side chain of Tyr 245 forms a hydrogen bond with the 2′-hydroxyl of $NADP^+$, which in turn may hydrogen-bond with the substrate carbonyl. Previous studies in related enzymes implicated this constellation in C_α protonation,[28,29] and we therefore constructed the corresponding Phe mutant. Interestingly, the Y245F mutant was unable to reduce either (*R*)- or (*S*)-perillaldehyde at detectable rates; however, its catalytic activity against 1-octen-3-one and *trans*-methyl 4-oxo-2-pentenoate was only modestly diminished. This behavior is inconsistent with our expectations for a residue serving as an obligatory general acid. Since no other obvious amino acid proton donors are found near C_α, we suggest that solvent or buffer species act directly as proton donors in rat Ltb4dh, analogous to the pathway followed by morphinone reductase.[30] As noted previously, there is sufficient room for solvent molecules near C_α in our modeled complexes.

The stereochemical courses of several alkene reductions have been uncovered, and enzymes mediating either *syn*- or *anti*-additions have been observed, although never both by a single biocatalyst. Like Ltb4dh, enoyl- and dienoyl-CoA reductases are members of the zinc-independent, medium-chain dehydrogenase superfamily that transfer hydride from NADPH to the substrate without a reduced flavin intermediate. Reynolds[31] and Anderson[32,33] have shown that these enzymes can follow many different stereochemical courses involving either net *syn*- or *anti*-addition of H_2, although a given enzyme followed only a single stereochemical course. Interestingly, as with Ltb4dh, it was not possible to identify an amino acid side chain in enoyl-CoA reductases that acts as a general acid, and it was suggested that C_α protonation involved transfer from solvent or buffer. Serra and co-workers investigated reductions of (*R*)- and (*S*)-perillaldehyde by baker's yeast, which afforded mixtures of *cis*-**2** and *trans*-**4**.[34] Deuterium labeling revealed that both net *syn*- and *anti*-additions of D_2 had occurred for both substrates. However, because whole yeast cells were used, it was not possible to determine whether a single reductase followed both stereo-

(27) We considered the possibility that NADPH occupied different binding sites for (*R*)- and (*S*)-perillaldehyde highly unlikely, given the many protein−cofactor contacts observed in the crystal structure of guinea pig Ltb4dh. These are likely conserved in the rat homologue and would strongly constrain the cofactor to its observed position.

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chemical pathways or if multiple reductases were involved. Because only a single enzyme was present in the present studies, this ambiguity is removed.

One unusual feature of Ltb4dh is its ability to catalyze both alkene and carbonyl reductions (though not in the same substrate), and it is possible that its stereochemical plasticity may be related to this property. Whether other members of the alkenal/one oxidoreductase subfamily within the medium-chain dehydrogenase/reductase superfamily can mediated both net *cis*- and *trans*-addition of H_2 remains to be demonstrated. This ability would be particularly useful in organic synthesis applications of these enzymes, as it would allow a larger diversity of chiral building blocks to be prepared.

Experimental Section

Isolation of GST-Ltb4dh. Plasmid pDJB19 was used to transform BL21(DE3). An overnight culture of the resulting strain was grown in LB medium containing 100 μg/mL ampicillin and diluted 1:100 into 500 mL of the same medium in a 2 L baffled flask. The culture was shaken at 37 °C until the OD_{600} was between 0.5 and 1.0, isopropyl thio-β-D-galactoside was added to a final concentration of 100 μM, and the culture was shaken for an additional 6 h at room temperature. Cells were collected by centrifugation, washed once with cold sterile water, and resuspended in 30 mL of 50 mM Tris-Cl, 4 mM $MgCl_2$, 2 mM KCl, 1 mM DTT, 1 mM PMSF, and 10% glycerol, pH 7.5 (loading buffer). All purification steps were carried out at 4 °C. Cells were lysed by passage through a French pressure cell, and debris was removed by centrifuging at 15000*g* for 20 min. The supernatant was passed through a 0.45 μm filter and loaded onto a column containing 10 mL of glutathione-uniflow resin (Clontech). The resin was thoroughly mixed with the lysate, and then it was allowed to settle in the column. The protein sample was continuously circulated through the column for 3−5 h using a peristaltic pump and a flow rate of *ca*. 0.5 mL/min. After this time, non-adsorbed lysate was drained, and the resin was washed in place with two 20 mL portions of loading buffer. The GST-Ltb4dh fusion protein was eluted by washing with 40 mL of freshly prepared elution buffer (loading buffer, 39.6 mL; 2 M NaOH, 0.40 mL; solid reduced glutathione, 310 mg). The eluant was concentrated by ultrafiltration (Amicon) and dialyzed overnight against 1 L of 20 mM Tris-Cl, 4 mM $MgCl_2$, 55 mM NaCl, 2 mM EDTA, 1 mM DTT, and 50% glycerol, pH 7.5. The purified fusion protein was stored at −20 °C. SDS−PAGE analysis showed one major band at the predicted molecular weight (observed 62.0 kDa; predicted 62.4 kDa).

Small-Scale Screening Reactions. Mixtures containing $NADP^+$ (0.20 μmol, 0.15 mg), glucose-6-phosphate (14 μmol, 4.7 mg), glucose-6-phosphate dehydrogenase (1.5 U, 5 μg), perillaldehyde (5 μmol, 0.75 mg), and the appropriate alkene reductase (GST-Ltb4dh or GST-OYE, 50−150 μg) in 1.0 mL of 100 mM KPi, pH 7.0, were incubated at 30 °C. Periodically, aliquots (100 μL) were extracted with an equal volume of EtOAc and analyzed by GC (30 m × 0.25 mm DB-17 column).

Isotopically Labeled Reactions. A 250 μL aliquot of 1 M Tris-Cl, pH 8.0, was evaporated to dryness by vacuum centrifugation (Speed-Vac), and then the residue was redissolved in the same volume of D_2O. This process was repeated, and then the solid was dissolved in 4.4 mL of D_2O. To this was added a solution of Ltb4dh (*ca*. 370 μg in 500 μL of H_2O) and solid TBADH (3.6 U, 3 mg, 1.21 U/mg) and $NADP^+$ (12 μmol, 9 mg). The reaction was initiated by adding a solution of the alkene [(*R*)- or (*S*)-perillaldehyde, 32 μmol, 5.0 μL) dissolved in *i*-PrOH-d_8 (650 μmol, 50 μL). After gentle shaking at room temperature for 16 h, the (*S*)-perillaldehyde reaction contained a trace of starting material, *cis*-**2** (*ca*. 20%), and *cis*-dihydroperillic alcohol (*ca*. 80%). Residual *cis*-**2** was converted to dihydroperillic alcohol by adding Ltb4dh (37 μg in 50 μL of H_2O), solid TBADH (1.2 U, 1.0 mg) and $NADP^+$ (5 μmol, 4 mg), and neat *i*-PrOH-d_8 (650 μmol, 50 μL) and gently shaking at room temperature for 24 h. The (*R*)-perillaldehyde reaction contained starting material (*ca*. 40%), *cis*-**2** (*ca*. 10%), and *cis*-dihydroperillic alcohol (*ca*. 40%) and minor amounts of unknown byproduct after gentle shaking at room temperature for 16 h. Complete conversion to *cis*-dihydroperillic alcohol was achieved in a total of 40 h by adding Ltb4dh (300 μg in 400 μL of H_2O), solid TBADH (1.2 U, 1.0 mg) and $NADP^+$ (5 μmol, 4 mg), and neat *i*-PrOH-d_8 (650 μmol, 50 μL). When complete, both reaction mixtures were extracted with $CDCl_3$ (8 mL), and then the organic fraction was dried with Na_2SO_4 and concentrated to *ca*. 800 μL under a nitrogen stream. ^1H and ^2H NMR spectra were recorded at 500 and 154 MHz, respectively.

Site-Directed Mutagenesis of Ltb4dh. Procedures for site-directed mutagenesis were adapted from published procedures.[35,36] Briefly, a mixture of two gel-purified PCR products encompassing the 5′- and 3′-ends of the Ltb4dh gene with the desired mutation (Y245F or Y262F) along with *Nco*I, *Hin*dIII-digested pDJB3 (ref 4) was used to transform chemically competent *E. coli* KC8 cells. *In vivo* recombinations occurred at both ends of the vector/insert junction and at the 37 bp overlap shared by the two PCR products centered on the codon undergoing mutation. Ampicillin-resistant colonies were screened by colony PCR, and positives were verified by DNA sequencing. After transformation of *E. coli* BL21(DE3) cells with the appropriate plasmids, mutant proteins were isolated in the same manner as wild-type Ltb4dh.

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Supporting Information Available: Map of plasmid pDJB19; aldehyde isomerization studies; NMR data for deuterated perillaldehyde reduction products; catalytic activity measurements of Y262F and Y245F mutants; GC data from enzymatic reductions of (*S*)- and (*R*)-**1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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