Mechanistic and Structural Studies of Apoform, Binary, and Ternary Complexes of the Arabidopsis Alkenal Double Bond Reductase At5g16970*

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Buhyun Youn‡, Sung-Jin Kim‡, Syed G. A. Moinuddin‡, Choonseok Lee‡, Diana L. Bedgar‡, Athena R. Harper‡, Laurence B. Davin‡, Norman G. Lewis§, and ChulHee Kang‡

From the ‡School of Molecular Biosciences, Washington State University, Pullman, Washington 99164-4660 and the §Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

In this study, we determined the crystal structures of the apoform, binary, and ternary complexes of the Arabidopsis alkenal double bond reductase encoded by At5g16970. This protein, one of 11 homologues in such as reduction of the 7–8-double bond of phenylpropanal substrates, is most closely related to the Pinus taeda phenylpropanal double bond reductase, involved in, for example, heartwood formation. Both enzymes also have essential roles in plant defense, and can function by catalyzing the reduction of the 7–8-double bond of phenylpropanal substrates, such as p-coumaryl and coniferyl aldehydes in vitro. At5g16970 is also capable of reducing toxic substrates with the same alkenal functionality, such as 4-hydroxy-(2E)-nonenal. The overall fold of At5g16970 is similar to that of the zinc-independent medium chain dehydrogenase/reductase superfamily, the members of which have two domains and are dimeric in nature, i.e., in contrast to their original classification as being zinc-containing oxidoeductases. As provisionally anticipated from the kinetic data, the shape of the binding pocket can readily accommodate p-coumaryl aldehyde, coniferyl aldehyde, 4-hydroxy-(2E)-nonenal, and 2-alkenals. However, the enzyme kinetic data among these potential substrates differ, favoring p-coumaryl aldehyde. Tyr-260 is provisionally proposed to function as a general acid/base for hydride transfer. A catalytic mechanism for this reduction, and its applicability to related important detoxification mammalian proteins, is also proposed.

The phenylpropanoid pathway, which is essentially restricted to vascular plants, is of increasing significance and general importance because of the extensive medicinal/health protecting properties of many of its derivatives. For example, this pathway results in formation of the potent antiviral agent, podophyllotoxin (1), which also serves as a semi-synthetic source of the highly successful cancer chemotherapeutic treatments, teniposide, etopoide, and Etopophos® (2–4). Other examples include: mataresinol and secoisolariciresinol, which are some of the dietary sources of the “mammalian” lignans, enterolactone/enterodiol, these being protective against the onset of various malignancies (5); the nordihydroguaiaretic acid derivatives, which show considerable promise against refractory cancers of the neck and the head (6); the potent antioxidant chlorogenic acid, which has well documented anticancer properties (7, 8), as well as reducing the risk of cardiovascular disease (9); and the monomeric allyl/proplyen phenols such as chavicol and eugenol, which have well known antibacterial and analgesic properties (10). Other medicinally important phenylpropanoid (acetate) pathway metabolites include dihydroconiferyl alcohol (1; Fig. 1), a potentially useful anti-inflammatory agent (11), and phlorizin (5), which shows considerable promise for treatment of diabetes mellitus, obesity, and stress hyperglycemia (12).

Our recent studies have been directed toward establishing the various biochemical pathways associated with formation of such metabolites, including defining the catalytic mechanisms and high-resolution structures of the participating pathway enzymes. Examples of these include pinoresinol/lariciresinol reductases (13, 14), secoisolariciresinol dehydrogenase (15–17), phenylcoumaran benzylic ether reductase (14, 18), isoflavone reductase (14), cinnamyl alcohol dehydrogenases (19, 20), and chavicol/p-anol and eugenol/isoeguenol synthases (21, 22), as well as dirigent proteins (in the presence of auxiliary oxidase capacity) (23–25). These studies are part of broader goals aimed toward (i) systematically engineering selected enzyme substrate binding pockets in terms of potentially modifying them to be more specific for a particular metabolite/metalidic pathway and (ii) better understanding how these pathways in plants have evolved.

The objective of the study described herein was to determine the mechanism and structures of the enzyme involved in formation of medicinally promising dihydrophenylpropanoid derivatives, such as dihydroconiferyl alcohol (1). In the Pinaceae, e.g. loblolly pine (Pinus taeda), various dihydrophenylpropanoids accumulate as heartwood-forming consti-

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The atomic coordinates and structure factors (codes 22JH, 22JH, 22JH, and 22JH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 To whom correspondence may be addressed: Inst. of Biological Chemistry, Washington State University, Pullman, WA 99164-6340. Tel.: 509-335-2605; Fax: 509-335-8206; E-mail: lewisn@wsu.edu.

2 To whom correspondence may be addressed: School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4660. Tel.: 509-335-1409; Fax: 509-335-9688; E-mail: chkang@wsunix.wsu.edu.
tuents, which contribute to the color, quality, and durability of its woody tissues; these can have either propanoic acid or propionaldehyde side chains (e.g. p-dihydrocoumaric (4)/dihydroferulic (3) acids and p-dihydrocoumaryl (2)/dihydro-
coniferyl (1) alcohols (Fig. 1) in *Picea glauca* (26)). Interestingly, their amounts (e.g. 1 and 2) are known to increase in the galls of *P. glauca* upon aphid attack (e.g. by *Adelges abietis*) (26) in further support of roles in plant defense.

In 2001, we reported the discovery of a *P. taeda* phenylpro-
penal (α,β double bond) reductase (PtPPDBR)3 (see Fig. 2A), for which the encoding gene (see Fig. 3) was cloned with the functionally recombinant protein obtained and characterized preliminarily (27). This enzyme, which is a member of the zinc-independent, medium chain dehydrogenase/reductase (MDR) superfamily, catalyzes the NADPH-dependent conversion of various monomeric and dimeric phenylpropanaldehydes (e.g. 6 and 7; see Fig. 2A) into the corresponding phenylpro-
aldehydes (e.g. 8 and 9). In terms of its amino acid similarity/identity, PtPPDBR has the closest homology to *Arabidopsis thaliana* (*AtDBR1*), as well as to a gene encoding (+)-pulegone reductase (PulR) from *Mentha piperita* (28), i.e. with similarities and identities of 63 and 43% and 62 and 44%, respectively (Fig. 3 and Table 1). In this study, we have described the characterization of the PtPPDBR homologue, *At5g16970* (AtDBR1), which catalysts the same conversions. By contrast, PulR from peppermint (*M. piperita*) (28) was not investigated; it is apparently specifically involved in a similar conversion of pulegone (10) to (+)-isomenthone (11) and (−)
menthone (12), with the latter predominating (Fig. 2B).

In the meantime, in related mammalian systems, there also emerged other important aspects of similar biochemical alkenal/alkenone reductions. These were associated with α,β double bond reductions of various keto/aldehydic moieties in both prostaglandin metabolism of guinea pig (*Cavia porcellus*) kidney tissue, e.g. where *13* is converted to *14* by 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase (12-HD/PGR) (Fig. 2C and Fig. 3) (29), and in rat liver

3 The abbreviations used are: PtPPDBR, *P. taeda* phenylpro-
penal (α,β double bond) reductase; AOR, alkenal/one oxidoreductase; AtDBR1, *A. thaliana* double bond reductase 1; EI-MS, electron impact mass spectra; ESI, electrospray ionization; GC-MS, gas chromatography-mass spectrometry; 12-HD/PGR, 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase; 4-HNA, 4-hydroxynonanal; 4-HNE, 4-hydroxy-(2E)-nonenal; HPLC, high pressure liquid chromatography; MDR, medium chain dehydrogenases/reductases; MES, 4-morpholinoethanesulfonic acid; PDB, Protein Data Bank; r.m.s.d., root mean square deviation; PulR, (+)-pulegone reductase; TBAF, tetrabutylammonium fluoride.

### Crystal Structure of Alkenal Double Bond Reductase

**FIGURE 1.** Dihydrophenylpropanoids (structures 1–4) and phlorizin (structure 5).

**FIGURE 2.** Reactions catalyzed by NADPH-dependent alkenal/alkenone/α,β double bond reductases in vitro. Reduction is shown of: A, p-coumaraldehyde (6) and coniferyl (7) aldehydes by PtPPDBR and AtDBR1; B, pulegone (10) by PulR in *Mentha piperita*; C, various α,β unsaturated ketones in prostaglandin biosynthesis (e.g. 13) by guinea pig leukotriene B4, 12-HD/PGR; D, 4-HNE (15) by AtDBR1.

**FIGURE 3.** Electron impact mass spectra of 12-HD/PGR reduction products. Reactions are shown of: A, 15-oxo-16-lipoxygenase A4 (13); B, 4-HNE (15); C, 15-oxo-lipoxygenase A4 (13) and 4-HNE (15) transformed by 12-HD/PGR; D, 4-HNE (15) transformed by AtDBR1.
Crystal Structure of Alkenal Double Bond Reductase

first described in 1995 (32). At that point, it was reported as being induced by oxidative stress and was considered then as a member of the \( \alpha/\beta \)-crystallin protein family based on 25–41% identity to mammalian \( \alpha \)-crystallins of unknown biochemical function (32, 33). Although this Arabidopsis protein was later shown capable of reducing diamide/quinone linkages (34), Mano et al. (35, 36) have since demonstrated that it can also reduce 4-HNE (15) and related potential substrates. This has led to a consideration that the Arabidopsis protein may, therefore, be involved in lipid-peroxidation derived alkenal reductions in response to oxidative stress.

Taken together, all of the above reports give an indication of the broad range of substrate versatility and thus of distinct potential biochemical functions that the double bond reductases can have. Such broad substrate versatilities have, however, resulted in significant confusion as to the actual range of physiological roles in vivo. Based on the demonstrated identification of PtPPDBR physiological function (27, 37), we can now characterize At5g16970 in comparative detail in terms of: (i) its substrate versatility (utilizing \( p \)-coumaryl (6)/coniferyl (7) aldehydes, and 4-HNE (15) as substrates in vitro), as well as the corresponding kinetic parameters; (ii) the crystal structures of the apo-, binary, and ternary complex forms for both \( p \)-coumaryl aldehyde (6) and 4-HNE (15) and the corresponding catalytic mechanism.

**EXPERIMENTAL PROCEDURES**

**Materials**—All solvents and chemicals used were reagent or HPLC grade unless otherwise stated. Chemical reactions were carried out under anhydrous conditions in a \( \text{N}_2 \) atmosphere using dry, freshly distilled solvents. Silica gel thin-layer chromatography (TLC) and silica gel column chromatography employed Partisil® PK5F (Silica gel 150 Å, 1 mm thickness), AL SIL G/UV254 (Whatman, 20 × 20 cm, 0.25 mm), and silica gel 60 (EM Science), respectively, with UV absorption and heating with phosphomolybdic acid reagent (2.5% in H\( _2 \)O) used for TLC plate visualization. NMR spectra were recorded on a Varian Mercury-Vx 300 MHz spectrometer operating at 300.1 (\( ^{1}H \))
and at 75.5 (13C), with chemical shifts given in δ ppm relative to tetramethylsilane and J values in Hz, respectively.

HPLC analyses employed an Alliance 2695 HPLC system equipped with a diode array detector (Waters, Milford, MA), and GC-MS analyses utilized an HP 5973 MS detector (electron impact mode, 70 eV), an HP 6890 GC system, and a 7673 series injector equipped with a RESTEK-SiI-MS (30 m × 0.25 mm × 0.25 μm) column. The carrier gas was helium with an initial flow of 1.4 ml min⁻¹ at a pressure of 11.65 p.s.i., with samples analyzed using the split injection mode and an injector temperature of 250 °C (split ratio, 10:1; split flow, 14.0 ml min⁻¹). The GC temperature program was initiated at 70 °C for 1 min, increasing to 170 °C at a rate of 8 °C/min, and held at 170 °C for 10 min. The mass range was scanned from m/z 50 to 800. HPLC electrospray ionization mass spectrometric analyses (LC-ESI-MS) were recorded on a Waters 2690 Alliance/Finnigan MAT LCQ, whereas electron impact mass spectra (EI-MS) were acquired on a Waters Integrity™ HPLC-MS system at an ionization voltage of 70 eV.

**Chemical Syntheses**—(E)-p-Coumaryl aldehyde (6) was synthesized exactly as described in Kim et al. (19), and coniferyl aldehyde (7) was from Aldrich.

For dihydroconiferyl aldehyde (9), to a solution of 4-O-tert-butylidimethylsilyl-(E)-coniferyl aldehyde (19) (500 mg, 1.71 mmol) in dry MeOH (10 ml) was added 10% palladium on activated charcoal (70 mg) with the resulting suspension stirred at room temperature under H2 for 6–8 h. The reaction mixture was then filtered, with the filtrate dried in vacuo. To the resulting 4-O-tert-butylidimethylsilyl dihydroconiferyl aldehyde derivative (450 mg, 1.53 mmol) dissolved in dry tetrahydrofuran (10 ml) under N2 at 0 °C was added a solution of tetrabutylammonium fluoride (TBAF) (1.0 M in tetrahydrofuran, 1.8 ml, 1.8 mmol) with the whole then allowed to stir for 45 min. The reaction mixture was quenched with a saturated NH4Cl solution (30 ml) and extracted with dry diethyl ether (50 ml × 2), the resulting combined organic solubles washed successively with water (30 ml × 2). The organic solubles were then dried (Na2SO4) and evaporated to dryness in vacuo. The residue so obtained was subjected to silica gel column chromatography (eluent: CHCl3/MeOH, 9:1), and then preparative silica gel TLC eluted with CHCl3/MeOH (9:1) to yield dihydroconiferyl aldehyde (9) (190 mg, 1.05 mmol, 70% yield). 1H NMR δ (300 MHz, CDCl3): 2.75 (2H, H-8), 2.89 (2H, H-7), 3.87 (3H, s, OMe), 6.66 (1H, d, J = 8.7, 2.3 Hz, H-6), 6.70 (1H, d, J = 2.3 Hz, H-2), 6.84 (1H, d, J = 8.7 Hz, H-5), 9.81 (1H, t, J = 1.67 Hz, CHO). EI-MS (70 eV) m/z 180 [M+1] (50), 137 (100).

4-HNE (15) was synthesized exactly as described by Gardner et al. (40) except for the purification/isolation steps. 4-HNE (15) was purified by silica gel preparative TLC using hexanes/diethyl ether (6:4) as eluants with detection by UV absorption and visualization of a bluish spot after staining and heating with phosphomolybdic acid reagent (2.5% (w/v) in H2O). The band corresponding to 4-HNE (15) was excised, eluted with acetone/diethyl ether (1:2, 30 ml × 2) with the combined organic solubles filtered and evaporated to dryness under N2 atmosphere by adding MeOH (1 ml) to avoid loss of 4-HNE (15) because of its volatility, and stored at −80 °C. 4-HNE (15) was obtained in ∼60–65% yield (46.7 mg, 0.3 mmol). GC-MS was employed next to determine the purity as well as the fragmentation pattern of 4-HNE (15) by preparing its trimethylsilyloxy derivative (41) using bis(trimethylsilyl)trifluoroacetamide-chlorotrimethylsilane (99:1 Supelco, 50 μl) and pyridine (20 μl). The following fragments were observed at a retention time of 13.10 min (90% pure) from the GC-MS analyses: m/z 228 [M+2]⁺, 213 [M⁺ - CH3], 199 [M⁺ - CHO], 157 [CHO-C6H5-CH2-OSi(CH3)3]+, 129 [CHO-C6H5-CH2-OSi(CH3)3]+ - CO, 73 [Si(CH3)3]+. The NMR spectra for HNE (15) were in close agreement with reported data (40, 42).

4-HNA (16) was synthesized via the diisobutylaluminium hydride reduction of γ-nonanoic lactone (43). The latter was synthesized by the Knoevenagel reaction of malonic acid with heptanal and lactonization with 85% sulfuric acid at 80 °C for 1 h (44, 45). The lactone (500 mg, 3.2 mmol) so formed was then reduced with a 1 M solution of diisobutylaluminium hydride in toluene (4.8 ml, 4.8 mmol) at −78 °C for 2 h exactly as described by Bloch and Gilbert (43). 4-HNA (16) was purified by silica gel preparative TLC using hexanes/diethyl ether (1:1) as eluants, with the band corresponding to 4-HNA (16) excised and eluted with acetone/diethyl ether (1:1, 30 ml × 2). The organic solubles were then combined, filtered, and dried under N2 atmosphere to afford 4-HNA (16), 420 mg, 2.65 mmol, 80–85% yield). GC-MS analyses of 4-HNA (16) showed the following fragments at retention time = 11.57 and 11.64 min corresponding to both S-(16a) and R-(16b) isomers (95% pure): m/z 230 [M⁺+2]⁺, 215 [M⁺ - CH3], 159 [CHO-C6H5-CH2-OSi(CH3)3]+, 73 [Si(CH3)3]+. The NMR spectrum for 4-HNA (16) was in close agreement with reported data (46, 47).

**Expression and Purification of AtDBR1—AtDBR1** (Atsg16970), cloned into an Invitrogen pTrcHis2-TOPO™ TA vector, was transformed into TOP10 Escherichia coli cells. Expression of AtDBR1 was induced by addition of isopropyl β-d-thiogalactopyranoside to a 0.5 mM final concentration at mid-log phase (A600 = 0.5). The induced cell suspension cultures were grown for 12 h at 25 °C with shaking at 250 rpm, with the cells subsequently harvested by centrifugation (3,000 × g for 20 min). The AtDBR1-derived pellet was suspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0, 10% glycerol), sonicated (5 × 10 s, model 450 Sonifer®, Branson Ultrasound), and centrifuged (20,000 × g for 40 min). After centrifugation, the supernatant was incubated with nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) for 1 h in an overhead shaker at 4 °C. After washing with 10 column volumes of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0), the fusion protein was eluted stepwise with 50 mM NaH2PO4, 300 mM NaCl, 100–300 mM imidazole, pH 8.0. Thereafter, the AtDBR1-enriched fraction was subjected to anion exchange column chromatography (Self Pack™ POROS® 10HQ, Applied Biosystems) pre-equilibrated
in Buffer A (50 mM Tris-HCl, pH 8.0, containing EDTA (1 mM) and dithiothreitol (1 mM)) at a flow rate of 3 ml min⁻¹. Recombinant AtDBR1 was eluted using a NaCl step gradient (to 0.1, 0.2, 0.5, and 2.0 M, 50 ml each) with the corresponding fractions of interest (eluting at 0.1 M NaCl) desalted and concentrated into Buffer B (20 mM Tris-HCl, pH 7.5) by ultrafiltration in an Amicon 8005 cell with a 10-kDa cutoff membrane (Millipore). This fraction was applied to a MonoQ™ GL10/100 anion exchange column (Amersham Biosciences) equilibrated in Buffer B at a flow rate of 2 ml min⁻¹ and eluted with a NaCl step gradient (0.05, 0.1, 0.2, 0.4, and 2.0 M; 20 ml each); the catalytically active AtDBR1 fraction eluted at 0.05 M NaCl. The AtDBR1 so obtained was concentrated with a final purity of >99% as estimated by SDS-PAGE (Coomassie Blue staining).

Kinetic Parameter Determinations—When p-coumaryl (6) and coniferyl (7) aldehydes were used as substrates, initial velocity kinetics were determined as follows. Assays consisted of MES buffer (100 mM, pH 6.25, 100 µl), 130 µl (3–8 µg of AtDBR1 purified as described in Kim et al. (19) in 20 mM Tris-HCl, pH 7.5, aldehydes 6 or 7 (10–0.1 mM, 10 µl), and NADPH (25 mM, 10 µl) in a total volume of 250 µl. Enzymatic reactions were initiated by the addition of AtDBR1, and after a 10-min incubation at 30 °C, they were stopped by the addition of glacial acetic acid (10 µl). An aliquot (80 µl) of each assay mixture was next subjected to reversed-phase HPLC analysis on a Symmetry Shield RP18 column (Waters; 150 × 3.9 mm inner diameter, 5 µm particle size) with the following elution conditions at a flow rate of 1 ml min⁻¹ and detection at 280 nm: the column was pre-equilibrated in a 5:95 ratio of CH₃CN (solvent A) and 3% AcOH in H₂O (solvent B). After introduction of the sample, this composition was held for 1 min, after which a linear gradient to A:B (5:95) over 30 min was carried out followed by a linear gradient to A:B (5:95) in 5 min, this being held for 1 min. The amounts of products 8 and 9 formed were determined using pre-established calibration curves.

Assays with 4-HNE (15) as substrate were carried out as described above but with 0.5 µg of AtDBR1 and an incubation time of 2 min. After addition of glacial acetic acid (10 µl), 4-hydroxybenzaldehyde (10 mM, 5 µl) was added as an internal standard, with the mixture extracted with diethyl ether (1 ml × 3). After vortexing, the diethyl ether layers were removed and combined, the ether solubles were dried (over Na₂SO₄), and the volume was reduced to ~100 µl under N₂, at which time 1,4-dioxane was added (100 µl). The resulting solution was transferred to a GC vial with the volume reduced to ~100 µl under N₂, and the trimethylsilyloxy derivative was prepared. The samples were next subjected to GC-MS analyses as described under “Chemical Syntheses.”

Molecular Mass Determination—Size exclusion chromatography/multiangle light scattering and dynamic light scattering were performed as described previously by Youn et al. (16, 20), with light scattering data acquired through accumulation (3 times) of 10 scans.

Crystallization of AtDBR1—For crystallization, a solution of purified AtDBR1 (48 mg ml⁻¹) in 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 1 mM dithiothreitol was prepared. Crystallization trials were performed using the hanging drop vapor diffusion method at two temperatures (277 and 293 K). ApoAtDBR1 crystals were obtained by mixing the above protein solution (1.5 µl) with an equal volume of reservoir solution containing 20% (w/v) polyethylene glycol 3350 and 0.2 M potassium chloride. Crystals usually appeared after 5 days, and larger crystals with dimensions of ~0.3 × 0.5 × 0.8 mm were obtained after 2 weeks. Although these crystals were fairly large, they were hollow and had a diffraction limit of 3.5 Å. Crystals were stabilized, and the diffraction limit was increased (up to 2.5 Å) by slowly adding concentrated buffer solution to the drops in which crystals were grown. For most crystals, the final buffer composition was 30% (w/v) polyethylene glycol 33500, 0.3 M potassium chloride. The crystals of AtDBR1 belong to the orthorhombic space group, P₂₁₂₁₂₁ (a = 49.46, b = 122.98, c = 148.00 Å), with two molecules in an asymmetric unit (Table 2). The binary complex (AtDBR1-NADP⁺) and the ternary complex (AtDBR1-NADP⁺-p-coumaryl aldehyde (6); ternary I) crystals were also produced under the same conditions except for the addition of 5 mM NADP⁺ and 5 mM p-coumaryl aldehyde (6) into the reservoir solution, respectively. Crystals of the other ternary complex (AtDBR1-NADP⁺-4-HNE (15); ternary II) were produced by soaking the binary complex crystal in a solution of 1 mM 4-HNE (15) (Alexis Biochemicals Inc.). All binary and ternary complexes crystallized in an orthorhombic space group, P₂₁₂₁₂₁, with corresponding unit cells of a = 49.15, b = 122.66, c = 147.94 Å (binary), a = 49.04, b = 122.54, c = 147.65 Å (ternary I), and a = 49.08, b = 122.45, c = 147.84 Å (ternary II), respectively. Data for the apoform (2.5 Å resolution), the binary complex (2.8 Å), and both of the ternary complexes (2.8 Å) were collected from the Berkeley Advanced Light Source (ALS, beam line 8.2.1/apoform and ternary I), Chicago Advanced Photon Source (APS, beam line NE-CAT/8-BMD/binary complex) and Rigaku Saturn 92/MicroMax-007 (Washington State University/ternary I) at a temperature of 100 K. Before freezing, the corresponding crystals were soaked for 5 min in cryoprotectant (25% glycerol in each reservoir solution).

Structural Solution and Refinement—The structure of apoAtDBR1 was solved by the molecular replacement method using a coordinate of the guinea pig (Cavia porcellus) leukotriene B₄ 12-HD/PGR (PDB code 1V3V) (29) and the software package AMoRe (48). The rigid body refinement of the initial position was carried out by using 15.0–3.0 Å resolution data and gave an R-value of 35%. After several cycles of positional and temperature factor refinements using the program X-PLOR (49) and a series of simulated annealing omit maps, most residues were fitted against the electron density. The binary and ternary complexes of AtDBR1 were again solved by the molecular replacement method but now using the apoAtDBR1 coordinates. The final R-factors (Table 2) for the apoform, as well as the binary and two ternary complexes of AtDBR1, were 19.7% (Rₓe = 24.3% for the random 5% data), 20.1% (Rₓe = 24.9% for the random 5% data), 18.9% (Rₓe = 23.1% for the random 5% data), and 19.9% (Rₓe = 24.1% for the random 5% data), respectively. The number of reflections above the 2σ level for the apoform was 30,000 (95% completeness) between 15.0 and 2.5 Å resolution. The crystals of the NADP⁺ binary complexes did not diffract as well as the apoform and gave reflection numbers of 21,151 (above 2σ, 95% completeness) between 15.0 and 2.8 Å resolution. In addition, the ternary complex data of both p-coumaryl...
aldehyde (6) and 4-HNE (15) were collected between 15.0 and 2.8 Å resolution, 20,191 and 20,187 (above 2σ), respectively. The root mean square deviations (r.m.s.d.) (from ideal geometry) of the final coordinates corresponding to the apoform and the binary, ternary I, and ternary II complexes were 0.014, 0.015, 0.024, and 0.025 Å for bonds and 3.2, 3.5, 4.5, and 4.6° for angles, respectively. All AtDBR1 coordinates have been deposited in the Protein Data Bank (apoform, 2J3H; binary complex, 2J3I; ternary complex with p-coumaryl aldehyde (6), 2J3J; ternary complex with 4-HNE (15), 2J3K).

RESULTS

It was first instructive to obtain the needed kinetic data for the recombinant (His-tagged) AtDBR1 using as substrates p-coumaryl aldehyde (6), coniferyl aldehyde (7), and 4-HNE (15), respectively. In this regard, all of the required substrates were synthesized as follows. (E)-p-Coumaraldehyde (6) was obtained in a 79% overall yield by the lithium aluminum hydride reduction of the 4-O-tert-butylmethylsilyl derivative of methyl-4-hydroxyacetophenone followed by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone and finally by deprotection with TBAF (19). (E)-Coniferylaldehyde (7) was purchased from Sigma-Aldrich, and the unstable 4-HNE (15) was synthesized by epoxidation of 3-(Z)-nonenal with 3-chloroperbenzoic acid followed by periodate oxidation and treatment with 1.3 M NaOH (40). The reduced products, p-dihydroconiferyl (8) and dihydroconiferyl (9) aldehydes, were individually synthesized via palladium on activated charcoal hydrogenation of the corresponding 4-O-tert-butylmethylsilyl aldehyde derivatives with subsequent deprotection using TBAF in ~60–70% yield (see “Experimental Procedures”). 4-HNA (16) was obtained via diisobutylaluminium hydride reduction of γ-nonanoic lactone to afford the desired product (R and S isomers) in an ~1:1 ratio as evidenced by GC-MS analyses. Additionally, a variety of monomeric phenylpropanoid substrate analogues and their dihydro products were prepared, e.g. cinnamyl (17)/5-hydroxyconiferyl (18)/sinapyl (19) aldehydes, the corresponding dihydrocinnamyl (30)/5-hydroxydihydroconiferyl (31)/dihydroxydiphenyl (32) aldehydes, as well as p-coumaric (20), caffeic (21), ferulic (22), 5-hydroxyferulic (23), and sinapic (24) acids and the potential dihydro-products (3, 4, 33–35). In an analogous manner, p-coumaryl (25), caffeil (26), coniferyl (27), 5-hydroxyconiferyl (28), and sinapyl (29) alcohols and the dihydro derivatives 1, 2, 36–38 were also synthesized (see Figs. 1 and 4 for structures). Each of the purified potential substrates (6, 7, 17–29) and products (1–4, 30–38) was then used to establish standard curves using HPLC, and GC-MS was used for 4-HNE (15)/4-HNA (16) analyses, i.e. to directly quantify substrate turnover and product formation. By contrast, all previous studies of 4-HNE (15) enzymatic reductions were carried out indirectly by measuring changes in UV absorption at 340 nm.

Kinetic Parameters/Substrate Versatility—With all of the corresponding potential substrates needed for the study in hand, the AtDBR1 cDNA was cloned into a pTrcHis-TOPO vector containing a N-terminal polyhistidine (His6) tag, with the plasmid used to transform E. coli TOP10 cells for gene expression. AtDBR1 was purified to apparent homogeneity (evaluated by SDS-PAGE with silver staining) following metal precipitation with 1.3 M NaOH (40). The reduced products, p-dihydroconiferyl (8) and dihydroconiferyl (9) aldehydes, were individually synthesized via palladium on activated charcoal hydrogenation of the corresponding 4-O-tert-butylmethylsilyl aldehyde derivatives with subsequent deprotection using TBAF in ~60–70% yield (see “Experimental Procedures”).
Crystal Structure of Alkenal Double Bond Reductase

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\begin{align*}
R_1 &= \text{CHO}; R_2R_3R_4 = \text{H}, \text{Cinnamyl aldehyde (17)} \\
R_1 &= \text{CHO}; R_2R_3 = \text{OH}; R_4 = \text{OCH}_3, 5-\text{OH Coniferyl aldehyde (18)} \\
R_1 &= \text{CHO}; R_2 = \text{OH}; R_3R_4 = \text{OCH}_3, \text{Sinapyl aldehyde (19)} \\
R_1 &= \text{COOH}; R_2 = \text{OH}; R_3 = \text{R}, \text{R} = \text{H}, \text{p-Coumaric acid (20)} \\
R_1 &= \text{COOH}; R_2 = \text{OH}; R_3 = \text{H}, \text{Cafeic acid (21)} \\
R_1 &= \text{COOH}; R_2 = \text{OH}; R_3 = \text{R}, \text{R} = \text{OCH}_3, \text{Ferulic acid (22)} \\
R_1 &= \text{COOH}; R_2R_3 = \text{OH}; R_4 = \text{OCH}_3, 5-\text{OH Ferulic acid (23)} \\
R_1 &= \text{COOH}; R_2 = \text{OH}; R_3R_4 = \text{OCH}_3, \text{Sinapic acid (24)} \\
R_1 &= \text{CH}_2\text{OH}; R_2 = \text{OH}; R_3R_4 = \text{H}, \text{p-Coumaryl alcohol (25)} \\
R_1 &= \text{CH}_2\text{OH}; R_2R_3 = \text{OH}; R_4 = \text{H}, \text{Cafley alcohol (26)} \\
R_1 &= \text{CH}_2\text{OH}; R_2 = \text{OH}; R_3R_4 = \text{H}, \text{R} = \text{OCH}_3, \text{Coniferyl alcohol (27)} \\
R_1 &= \text{CH}_2\text{OH}; R_2 = \text{OH}; R_3R_4 = \text{OH}, R_4 = \text{OCH}_3, 5-\text{OH Coniferyl alcohol (28)} \\
R_1 &= \text{CH}_2\text{OH}; R_2 = \text{OH}; R_3R_4 = \text{OCH}_3, \text{Sinapyl alcohol (29)} \\
R_1 &= \text{CHO}; R_2R_3R_4 = \text{H}, \text{Dihydrocinnamyl aldehyde (30)} \\
R_1 &= \text{CHO}; R_2R_3 = \text{OH}; R_4 = \text{OCH}_3, 5-\text{OH Dihydroconiferyl aldehyde (31)} \\
R_1 &= \text{CHO}; R_2 = \text{OH}; R_3R_4 = \text{OCH}_3, \text{Dihydrocinamyl aldehyde (32)} \\
R_1 &= \text{COOH}; R_2R_3 = \text{OH}; R_4 = \text{H}, \text{Dihydor caffeic acid (33)} \\
R_1 &= \text{COOH}; R_2R_3 = \text{OH}; R_4 = \text{OCH}_3, 5-\text{OH Dihydroferulic acid (34)} \\
R_1 &= \text{COOH}; R_2 = \text{OH}; R_3R_4 = \text{OCH}_3, \text{Dihydrosinapic acid (36)} \\
R_1 &= \text{CH}_2\text{OH}; R_2R_3 = \text{OH}; R_4 = \text{H}, \text{Dihydorcaffeal alcohol (36)} \\
R_1 &= \text{CH}_2\text{OH}; R_2 = \text{OH}; R_3R_4 = \text{OH}, R_4 = \text{OCH}_3, 5-\text{OH Dihydroconiferyl alcohol (37)} \\
R_1 &= \text{CH}_2\text{OH}; R_2 = \text{OH}; R_3R_4 = \text{OCH}_3, \text{Dihydrosinapyl alcohol (38)}
\end{align*}
\]

FIGURE 4. Phenylpropanoids (structures 17–29) and dihydrophenylpropanoids (structures 30–38).

Table 3 summarizes the kinetic data so obtained. In our hands, both p-coumaryl (6) and coniferyl (7) aldehydes served as the most efficient substrates (\(k_{\text{cat}}/K_m\) of 5360 and 1060 M\(^{-1}\) s\(^{-1}\)) relative to that of 4-HNE (15), which was less efficiently utilized (600 M\(^{-1}\) s\(^{-1}\)). Additionally, several other substrates were evaluated for their capacity to undergo comparable alkenal (\(\alpha,\beta\) double bond) reductions, including cinnamyl (17), 5-hydroxyconiferyl (18), and sinapyl (19) aldehydes. However, none of these closely related substrate analogues was converted into the corresponding dihydro derivatives (30–32), indicating that the substrate versatility of the AtDBR1 was fairly limited. This observation contrasts with reports that the AOR from rat liver is capable of reducing the \(\alpha,\beta\) unsaturated double bond of cinnamyl aldehyde (17) along with a very broad range of potential substrates; however, in that study the presumed conversion of 4-HNE (15) and 4-HNA (16) was confirmed by direct GC-MS analyses of amounts of both 4-HNE (20) and 4-HNA (21) utilizing (600 M\(^{-1}\) s\(^{-1}\)) as the most efficient substrates, with no conversion of the corresponding dihydro product (30–32). In an analogous manner, none of the hydroxycinnamic acids (20–24) were converted into the dihydrocinnamates (3, 4, 33–35) by AtDBR1, nor were any of the corresponding alcohols (25–29) directly reduced to afford 1, 2, 36–38.

As anticipated, 4-HNE (15) was also readily reduced, as evidenced by direct GC-MS analyses of amounts of both 4-HNE (15) and the enzymatic product 4-HNA (16) in the assay mixtures. However, these assays revealed that although 4-HNE (15) had a slightly lower \(K_m\) than p-coumaryl (6)/coniferyl (7) aldehydes, the overall \(V_{\text{max}}\) was significantly lower leading to a decreased catalytic efficiency. Interestingly, Mano et al. (35) had reported a much lower \(K_m\) (by indirect detection), as was also noted for the indirect measurement of cinnamaldehyde (17) reduction by Dick et al. (30). In our hands, such an efficient conversion of 4-HNE (15) into 4-HNA (16) could not be duplicated, nor was cinnamaldehyde (17), reduced as indicated above. Based on the data presented herein, we therefore concluded that p-coumaryl aldehyde (6) serves as the best in vitro substrate. The data so obtained also revealed that for effective catalytic activity, the double bond must be in conjugation with an aldehydic moiety, although the substrate versatility was fairly limited.

Overall Structure of AtDBR1—To understand in greater detail the mechanistic/structural basis for the AtDBR1 kinetic properties, the recombinant AtDBR1 was next crystallized in its apoform and NADP\(^+\) binary and ternary complexes using the two different substrates p-coumaryl aldehyde (6) and 4-HNE (15), respectively. Crystals of both ternary complexes were obtained by mixing AtDBR1 with NADP\(^+\)/p-coumaryl aldehyde (6) and by soaking the AtDBR1/NADP\(^+\) binary complex crystals in a solution containing 4-HNE (15), respectively. The apoform of AtDBR1 was determined at 2.5 Å resolution by the molecular replacement method using coordinates of the aforementioned 12-HD/PGR (PDB code 1V3V) from guinea pig (C. porcellus) (29), which shows 56/41% similarity/identity to AtDBR1 (Table 1). Additionally, the binary and ternary complex structures of AtDBR1 were determined at 2.8 Å resolution using the coordinates of the deduced structure of the AtDBR1 apoform.

As shown in Fig. 5, AtDBR1 is a homodimer with two subunits arranged through a noncrystallographic 2-fold axis. The two subunits are virtually superimposable, with an r.m.s.d. of 0.67 Å between the corresponding Ca\(^{\alpha}\) atoms of the two subunits, without including the residues from 60 to 70 in the case of the apoform. The loop region of residues 60–70 connecting the \(\alpha_1\) and \(\beta_4\) regions is disordered in one subunit, whereas the corresponding area of the other subunit is ordered because of crystal packing interactions. Both static and dynamic multiangle laser light-scattering data of AtDBR1 confirmed its dimeric nature (Fig. 6, A and B).
The subunit dimerization is achieved mainly through interactions between the two β-strands from each subunit (8F) in an anti-parallel manner, thereby forming an extended 12-stranded β-sheet across the dimer interface (Fig. 5). Another smaller dimer-forming interaction is at the opposite side of this βF strand interaction and involves two hydrogen bonds between the two pseudo 2-fold related Val-268 residues (not shown). There are additional salt bridges and hydrogen bonds between the side chains and between the side chain and the main chains of individual subunits.

The overall fold of AtDBR1 belongs to that of the zinc-independent MDR superfamily. Like other MDRs, each monomer is composed of two domains: a substrate-binding domain (residues 1–137 and 306–345) and a nucleotide-binding domain (Rossman fold, residues 138–305) (Figs. 3 and 5). The substrate-binding domain has three α-helices and ten β-strands forming two β-sheets, one of which is a highly twisted, eight-stranded, β-barrel-like structure as observed previously for the putative NADP-dependent oxidoreductase from Mus musculus (Fig. 3; PDB code 1VJ1) (50). The nucleotide-binding domain of AtDBR1 also has seven α-helices and six β-strands forming a typical six-stranded parallel β-sheet flanked by three helices on each side. In the binary and ternary complexes, the corresponding NADP⁺ and substrates were located at the active site clefts between the substrate- and nucleotide-binding domains, as described in detail below. Upon NADP⁺ and/or substrate binding, no significant changes were detected in either overall conformation or of the amino acids in the binding pockets as reflected in small r.m.s.d. values of 0.42–0.66 Å (not shown) among the Ca atoms of the apoform and the binary and ternary complexes. A minor positional change in the backbone was observed in the area of residues 36–39, which connects β2 and β3.

A Dali search (53) of the PDB data base indicated that the highest match was to the 12-HD/PGR from guinea pig C. porcellus (PDB code 1V3V) with a Z-score of 43.0; this was followed by several quinone reductases, including the human ζ-crystalline (PDB code 1YB5) with a Z-score of 32.3 (not shown), and the putative NADP-dependent oxidoreductase from M. musculus (1VJ1) of 31.4. The latter two show only ~30 and 19% identity, however, to AtDBR1, based on amino acid sequence comparisons. Nevertheless, all of these high Z-scored proteins belong to the zinc-independent MDR superfamily.

The sequence alignment among the above-mentioned enzymes (Fig. 3) also revealed that AtDBR1 has several areas of deletion and insertion when compared with the other enzymes, which include several loops and even some secondary structural elements. Especially, one loop region that is composed of residues 30–40 connecting β2 and β3 is quite different, particularly when compared with the mammalian enzymes (Fig. 3). In addition, a highly disordered loop region (amino acids 60–89) in the structure of AtDBR1 is not aligned well among the other enzymes.

In general, however, all of the enzymes show a high degree of sequence similarity for most of the nucleotide-binding domain up to the conserved 255GXGS258 motif; this is known to stabilize both the adenine and nicotinamide moieties of the cofactor in the NADPH-bound form of quinone oxidoreductase (50). On the other hand, the remaining part of the nucleotide-binding domain (residues 262–305) has a low level of sequence similarity and participates mainly in subunit interactions.

**NADP⁺-binding Site**—As seen for other members of the MDR superfamily, the electron density corresponding to NADP⁺ in AtDBR1 was located in the cleft between the two domains formed by the carboxyl ends of βA, βB, BD, βE, βF, and the loop connecting βF and G. There was also a clear and continuous electron density in the initial Fᵣ – Fₑ map (Fig. 7A, inset); all of the side chains around this cofactor were thus in a well defined electron density that is highly conserved among the other enzymes.

**Ternary complex of AtDBR1 homodimer with bound NADP⁺/p-coumaryl aldehyde (structure 6) (A) and NADP⁺/4-HNE (structure 15) (B).** The substrate- and nucleotide-binding domains are depicted in green and violet in the lower subunit, and in brown and light blue in the upper subunit, respectively. NADP⁺ is shown in dark blue, with p-coumaryl aldehyde (6) or 4-HNE (15) in dark brown. The active site in the lower subunit is outlined by a dotted box, and the secondary structure elements of AtDBR1 are numbered sequentially as α1–α3/αA–αG and β1–β10/βA–βF.
hydrogen bonding distance of the amide moiety of the nicotinamide ring (Fig. 7A). Interestingly, the above-mentioned

\[ \text{CGXXSXY} \]

motif, which is also located near the nicotinamide ring, can be extended to “CGXXSXY” among the compared zinc-independent MDR enzymes. The net effect is that the freedom of the nicotinamide ring is very restricted, presumably fixing the position of the C-4 atom during catalysis.

Notably, both the hydroxyl group of the Tyr-260 and the backbone nitrogen of Tyr-53, which are completely conserved among all of the oxidoreductases compared (see Fig. 3), are within hydrogen bonding distance of the O-2' of the nicotinamide ribose ring. In contrast to the well anchored nicotinamide ring, however, the adenine ring does not have much interaction with the surrounding amino acids and thus should be relatively free to move, as indicated by its high B-value.

The AtDBR1, NADP⁺, and \( p \)-Coumaryl Aldehyde (6) Ternary Complex—In the ternary complex, the observed distances between the C-4 atom of the nicotinamide ring and the \( \beta \)-carbon of the \( \alpha \beta (7,8) \)-unsaturated double bond of \( p \)-coumaryl aldehyde (6) in the two subunits are 3.8 and 4.0 Å, respectively. Thus, \( p \)-coumaryl aldehyde (6), located at the active site cleft between the substrate- and nucleotide-binding domains, is in the proper orientation to the nicotinamide ring for the well established A-face-specific hydride transfer from C-4 to the corresponding substrate reaction center (Fig. 7B).

Unlike the NADP⁺ molecule deeply buried inside the binding pocket with many interactions involving various amino acid residues, \( p \)-coumaryl aldehyde (6) is relatively exposed to the solvent; thus a weaker binding constant can be expected when compared with that of the cofactor. The inner wall of this exposed substrate-binding site is also surrounded by the nicotinamide ring, as well as Tyr-53, Tyr-81, Met-138, Tyr-260, Ser-287, and Tyr-290 from one subunit and Ile-275 and Tyr-276 from another, indicative of its quite polar nature (Fig. 7B). In either the apoform or the NADP⁺ binary complex, however, this substrate-binding site is filled with water molecules, reflecting its somewhat hydrophilic nature.

Of particular note is the fact that the phenolic hydroxy group of Tyr-260 is within hydrogen bonding distance to the aldehydic oxygen of the substrate (6), in addition to the \( 2' \)-hydroxyl group of the nicotinamide ribose described previously. Therefore, we considered that this conserved Tyr-260 residue is hydrogen bonded to both. In addition, the Tyr-81 hydroxyl group is also potentially within hydrogen bonding distance of the phenolic hydroxyl group (Fig. 7B), whereas the Ser-287 hydroxyl group is \(~\sim 3.9 \text{ Å} \) away, thus probably too far away for hydrogen bond formation. However, because of their location in the flexible (high temperature factor) loops, there provisionally remained a possibility that hydroxyl groups in Tyr-81 and Ser-287 were potentially involved in facilitating substrate binding.

Lastly, the phenol ring of the highly conserved Tyr-53, which is located between a relatively conserved short loop and one-turn \( \alpha \)-helix, is in a potential stacking interaction with the corresponding phenol ring of the bound \( p \)-coumaryl aldehyde (6), thereby probably further facilitating orientation of the substrate within the specificity pocket (Fig. 7B).
The AtDBR1, NADP⁺, and 4-HNE (15) Ternary Complex—A well defined density for 4-HNE (15) was also located in the same position as for p-coumaryl aldehyde (6) (Fig. 7C, inset). As a result, the corresponding conformation of the individual side chain and NADP⁺ did not change between the two ternary complexes. As observed in the p-coumaryl aldehyde (6) ternary complex, Tyr-260 is also within hydrogen bonding distance to the aldehydic oxygen of 4-HNE (15) and to the 2'-hydroxyl group of the nicotinamide ribose (Fig. 7C). The distances between the C-4 atom of the nicotinamide ring and the β-carbon of the α,β-unsaturated double bond of 4-HNE (15) in the two subunits are 3.8 and 4.0 Å, respectively, which are approximately the same distances as for the p-coumaryl aldehyde (6) complex. Moreover, as in the ternary complex with p-coumaryl aldehyde (6), the conserved Tyr-53 is now in a hydrophobic interaction with the corresponding aliphatic chain of the bound 4-HNE (15), thereby again facilitating orientation of the substrate within the specificity pocket (Fig. 7C).

DISCUSSION

In contrast to two other oxidoreductases involved in related aspects of phenylpropanoid metabolism, i.e. secoisolariciresinol dehydrogenase (15–17) and cinnamyl alcohol dehydrogenase 5 (19, 20), the crystal structure of AtDBR1 shows that it belongs to the Zn²⁺-independent MDR family. This class of enzymes, as indicated above, includes PtPPDBR, PulR, 12-HD/PGR, AOR, the putative NADP-dependent oxidoreductase from M. musculus (PDB code 1VJ1), and the ζ-crystallins. The enzymatic reaction mechanism of this class of enzymes that lack Zn²⁺ is, however, still unclear, e.g. when compared with the very well established Zn²⁺-containing MDRs such as alcohol dehydrogenase. Indeed, a majority of the enzymes provisionally belonging to this group are still depicted as putative gene products (55).

In terms of the overall distribution of secondary structural elements and the local active site, AtDBR1 displays a striking similarity to 12-HD/PGR. As discussed earlier, the latter catalyzes the reduction of a very broad...
array of \( \alpha, \beta \)-unsaturated ketones and aldehydes, including toxic products of lipid peroxidation in addition to that of the 13–14-double bond of 15-oxoprostaglandins (29, 56). Accordingly, most of the residues constituting the NADP(H)-binding sites are very similar between AtDBR1 and 12-HD/PGR. Indeed, the overall shapes of the binding pockets for the cofactor are also very similar, and the bound cofactor in both reductases adopts the same sugar conformation.

For guinea pig 12-HD/PGR and rat liver AOR, a ketoreductase reaction mechanism has been proposed with a conjugated enolate as the reaction intermediate (29, 55). Apparently, the latter enzyme is also able to reduce one of the phenylpropanoids, cinnamyl aldehyde (17) (30), whereas AtDBR1 does not reduce it. It is tempting to speculate, therefore, that the hydrophobic nature of the phenyl moiety of the cinnamyl aldehyde (17) can be accommodated in the hydrophobic substrate-binding pocket of 12-HD/PGR but not in the AtDBR1 binding pocket, which is more hydrophilic in nature. In addition, the intrinsic difference in terms of rotational freedom around the C-1–C-7 bond between cinnamyl aldehyde (17) and \( \)p-coumaryl aldehyde (6) (resulting from an ability of the latter to more readily form resonance hybrids because of the phenol functionality at C-4) may also provisionally explain the substrate preferences in binding and catalysis between 12-HD/PGR and AtDBR1.

Yet, in contrast to the similarities between the 12-HD/PGR and AtDBR1, the previously proposed candidate catalytic residue (Tyr-262) for the former could not be further substantiated. In part, this is because this residue (Tyr-276 in AtDBR1) is not conserved in the other sequences (see Fig. 3), and from the analysis of the crystal structure, it is too far away (5.5–5.9 Å) in AtDBR1 to be able to hydrogen bond with the aldehydic group of the substrate.

On the other hand, the residue Tyr-260 is conserved in all enzymes shown in Fig. 3. Hence, from the observed hydrogen bonding pattern and the strong conservation of Tyr-260 among related enzymes, we propose that this residue serves as a general acid/base by stabilizing the enol form of the transition state (Fig. 8, A and B). Tyr-260 is also apparently hydrogen bonded to the 2'-hydroxyl group of the nicotinamide ribose, thereby potentially enabling the \( \)pK\text{a} of its hydroxyl group to be modulated further. A similar catalytic mechanism can also be envisaged for 12-HD/PGR, PtPPDBR, PulR, AOR, and the putative NADPH-dependent oxidoreductase from \( \)M. musculus (1VJ1).

From our studies using \([4^R-3H]\)- and \([4^S-3H]\)NADPH, AtDBR1\(^5\) and PtPPDBR (37) catalyze the transfer of the pro-\( R \) hydride from NADPH to that of the double bond between C-7 and C-8, with this bond being polarized due to its neighboring carbonyl group. In agreement with this finding, the observed interactions between the carbonyl oxygen of both substrates, \( p \)-coumaryl aldehyde (6) and 4-HNE (15), and the Tyr-260 residue can also potentially stabilize the transient oxygen enolate intermediate, thereby facilitating pro-\( R \) hydride transfer from the C-4 atom of the NADPH nicotinamide to the substrate (Fig. 8, A and B). As anticipated, however, AtDBR1 was not able to reduce the corresponding unpolarized double bond of the various phenylpropenols 25–29, as shown from our kinetic data.

Overall, we can propose a concerted catalytic mechanism for hydride transfer to carbon 7 of \( p \)-coumaryl aldehyde (6) (C-3 in 4-HNE (15)), this ultimately being followed by protonation (of

\(^5\)S.-J. Kim, S. G. A. Moinuddin, D. L. Bedgar, L. B. Davin, and N. G. Lewis, unpublished observations.

FIGURE 8. Observed potential interactions in the ternary complex of AtDBR1 with NADP\(^{+}\)/\( p \)-coumaryl aldehyde (structure 6) (A), NADP\(^{+}\)/4-HNE (structure 15) (B), and their corresponding schematic reaction mechanisms (C and D). Arrows indicate the stacking interaction between phenol rings. The phenolic group of Tyr-81 is within a hydrogen bond distance of the \( p \)-coumaryl aldehyde (6) phenolic group, and the phenolic group of 4-HNE (15) is within hydrogen bonding distance of the carbonyl group of both bound \( p \)-coumaryl aldehyde (6) and 4-HNE (15).
C-8 in p-coumaryl aldehyde (6) and of C-2 in 4-HNE (15)) (Fig. 8, C and D). Indeed, because there is no adjacent amino acid in AtDBR1 able to donate a proton to C-8, this proton is presumed to be from the solvent. As mentioned above and shown in Fig. 7, the substrate-binding site of AtDBR1 is well exposed to the solvent. Considering the fact that one side wall of the substrate-binding site is made up of a nicotinamide ring of the tightly bound cofactor, the binding of either p-coumaryl aldehyde (6) or 4-HNE (15) can presumably be facilitated by cofactor binding. Therefore, it is reasonable to speculate that NADPH binds first and NADP+ leaves last, indicating that the kinetic mechanism of AtDBR could be an ordered Bi-Bi or Theorell-Chance mechanism, as in the cases of 12-HD/PGR and AOR (55, 57). The dissociation of the NADP+ could, however, be rate-limiting.

In addition, the C-7 = C-8 unsaturated double bond of p-coumaryl aldehyde (6) is in conjugation with both the allylic aldehyde moiety and the aromatic ring. Accordingly, the highly conserved Tyr-53 of AtDBR1 is thus possibly in a stacking position with the phenolic ring of the substrate. Therefore, \( \pi-\pi \) interactions between the phenolic substrate (6) and the nearby Tyr-53 residue could potentially stabilize further the propenal transition state, i.e. by withdrawing electron density away from C-8 (Fig. 8, A and C). This potential interaction may also explain why AtDBR1 can catalyze p-coumaryl aldehyde (6) reduction more efficiently than 4-HNE (15), as indicated from the kinetic data (Table 3).

CONCLUDING REMARKS

With the discovery of the physiological function of PtPPDBR, it was possible to study the overall substrate versatility and catalytic mechanism of Arabidopsis At5g16970 (AtDBR1). This established first that p-coumaryl (6)/coniferyl (7) aldehydes were the preferred substrates over that of 4-HNE (15). Additionally, it identified Tyr-260 (i.e. in AtDBR1) as responsible for general acid/base catalysis and thus enabled us to define the overall catalytic mechanism of the alkenal double bond reductases in vascular plants. These findings also provide a much fuller understanding of the catalytic mechanism of the more distantly related mammalian double bond reductase homologues such as 12-HD/PGR and AOR.

Careful inspection of the AtDBR1 substrate-binding site in both the apoform and the ternary complex also revealed that it has several factors favoring p-coumaryl aldehyde (6) as a substrate, even though it can accommodate (and reduce) other phenylpropanoids and 2-alkenals of longer/shorter chains. For example, because the substrate binding pocket is polar in nature, the presence of hydroxyl groups in p-coumaryl (6)/coniferyl (7) aldehydes, as well as in 4-HNE (15), enhance their binding affinities. By contrast, similar size molecules, such as cinnamyl aldehyde (17), which lacks a phenolic/hydroxyl group, have a much lower affinity for the pocket as suggested by the lack of activity. Additionally, although not depicted in the proposed catalytic mechanism, the phenolic group of p-coumaryl aldehyde (6) is fully conjugated to the allylic aldehydic moiety, and this (because of a potentially reduced level of rotational freedom in the C-1–C-7 bond of cinnamyl aldehyde (17) versus p-coumaryl aldehyde (6)) may provide a further rationale as to why 17 does not serve as a substrate. Finally, the relatively tight fit of the overall binding pocket might also serve to explain why more highly substituted potential substrates, such as 5-hydroxyconiferyl (18) and sinapyl (19) aldehydes are unable to undergo reductive conversions (i.e. when the phenolic group at C-4 is flanked by \( \alpha \) substituents). Future work will be directed toward more comprehensively establishing the range of physiological functions of AtDBR1 and PtPPDBR, respectively.

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