NADPH-dependent alkenal/one oxidoreductase (AOR) from the rat is a phase 2/antioxidative enzyme that is known to catalyze the reduction of the carbon-carbon double bond of α,β-unsaturated aldehydes and ketones. It is also known for its leukotirene B4 12-hydroxydehydrogenase activity. In order to begin to understand these dual catalytic activities and validate its classification as a reductase of the medium-chain dehydrogenase/reductase family, an investigation of the mechanism of its NADPH-dependent activity was undertaken. Recombinant AOR and a 3-nonen-2-one substrate were used to perform steady-state initial velocity, product inhibition, and dead end inhibition experiments, which elucidated an ordered Thorell-Chance kinetic mechanism with NADPH binding first and NADPH* leaving last. A nearly 20-fold preference for NADPH over NADH was also observed. The dependence of kinetic parameters V and V/K on pH suggests the involvement of a general acid with a pK of 9.2. NADPH isomers stereospecifically labeled with deuterium at the 4-position were used to determine that AOR catalyzes the transfer of the pro-R hydride to the β-carbon of an α,β-unsaturated ketone, illudin M. Two-dimensional nuclear Overhauser effect NMR spectra demonstrate that this atom becomes the R-hydride at this position on the metabolite. Using [4R,2H]NADPH, small primary kinetic isotope effects of 1.16 and 1.73 for V and V/K, respectively, were observed and suggest that hydride transfer is not rate-limiting. Atomic absorption spectroscopy indicated an absence of Zn2+ from active preparations of AOR. Thus, AOR fits predictions made for medium-chain reductases and bears similar characteristics to well known medium-chain alcohol dehydrogenases.

NADPH-dependent alkenal/one oxidoreductase (AOR)1 is an enzyme that reduces the carbon-carbon double bond of a variety of α,β-unsaturated aldehydes and ketones (1). It is coregulated in the rat with a variety of phase 2/antioxidative enzymes, including NAD(P)H:quinone reductase, glutathione S-transferases, and UDP-glucuronosyltransferases, through the Keap1/Nrf2 signaling pathway (2). α,β-Unsaturated aldehydes and ketones are electrophilic and capable of reacting, via a Michael addition mechanism, with important cellular nucleophiles, which in turn leads to macromolecular (protein, DNA) dysfunction and cell death. Because saturated carbonyls lack this reactive moiety, they are often far less toxic. Thus, hydrogenation of the α,β-double bond by AOR conceptually results in detoxication (1, 3).

Of the AOR substrates identified, several are common environmental pollutants (methyl vinyl ketone and acrolein) or products of lipid peroxidation (4, 5). The latter process involves reaction of oxygen free radicals with polyunsaturated fatty acids to form aliphatic α,β-unsaturated aldehydes such as 4-hydroxy-2-nonenal (4HNE), 2-hexenal, and 2,4-decadienal (5). These reactive molecules probably mediate many of the detrimental effects of oxidative stress. 4HNE is extremely cytotoxic, an abundant product of lipid peroxidation, and an excellent substrate of AOR (1). Cells engineered to overexpress AOR are resistant to 4HNE-mediated cell death and protein adduct formation (1). Thus, the physiological antioxidant role of the NADPH-dependent activity of AOR was elucidated.

Several additional endogenous substrates for this activity have been identified including the 15-oxoprostaglandins (6), 15-oxoolipoxin A4 (7), and members of the prostaglandin J2 family.2 Whereas the former two classes of compounds are inactive metabolites, 15-deoxy-Δ12,14-prostaglandin J2 is thought to be an endogenous activator of the Nrf2 transcription factor (8). Recently, AOR was also found to reduce the 8,9-double bond of the mycoxin illudin S and M (9). Irofulven, an illudin S derivative and promising anti-cancer alkylating agent, was also found to be a substrate. Paradoxically, the metabolism of irofulven by AOR was found to activate its cytotoxic nature by unmasking an electrophilic cyclopropyl group.

Interestingly, AOR was first isolated because of its NADP+- dependent alcohol dehydrogenase activity (10). Via oxidation of the 12-hydroxyl group, AOR is capable of inactivating the potent neutrophil chemoattractant leukotriene B4 (LTB4). This is not simply the reverse of NADPH-driven hydrogenation but rather a distinct catalytic activity that may occur due to a reverse substrate orientation. LTB4 is the only known substrate for this activity. Rates of NADPH-dependent α,β-double bond reduction are commonly several orders of magnitude greater than that measured for the NADP+-dependent oxidation of LTB4.

Little is known of the structure of AOR. It bears similar

1 The abbreviations used are: AOR, NADPH-dependent alkenal/one oxidoreductase; 4HNE, 4-hydroxy-2-nonenal; MDR, medium chain dehydrogenase/reductase; LTB4, leukotriene B4; LADH, liver alcohol dehydrogenase; NOESY, nuclear Overhauser effect NMR spectroscopy.

2 R. A. Dick and T. W. Kensler, unpublished results.
characteristics and sequence homology to members of the medium-chain dehydrogenase/reductase (MDR) superfamily, which includes liver alcohol dehydrogenase (LADH), \( \zeta \)-crystallin, and Escherichia coli quinone reductase, and has been classified as such (11). It is closely related to a MDR subfamily of oxidoreductases that include several putative microbial enzymes (12, 13) and one cloned and characterized NADPH: quinone oxidoreductase, P1-\( \zeta \)-crystallin, from Arabidopsis thaliana (14). This group is designated COG2130 in the clusters of orthologous groups data base (available on the World Wide Web at ncbi.nlm.nih.gov/COG). P1-\( \zeta \)-crystallin is capable of catalyzing one-electron reductions of quinones and two-electron reductions of azo-carbonyls and \( \alpha, \beta \)-unsaturated aldehydes and ketones, including 4HNE (15, 16). It is also up-regulated employed inhibitor concentrations of 0, 50, 150, and 250 \( \mu \)M to determine its mode of inhibition versus NADPH. Data were fit to one the following equations using GraFit 5.0.

\[
V = \frac{V_{\text{max}} [S]}{K_v + [S]} + \frac{V_{\text{max}}}{K_v}
\]

(Eq. 3)

\[
V = \frac{V_{\text{max}} [S]}{K_v + [S]} + \frac{V_{\text{max}}}{K_v + [S]}
\]

(Eq. 4)

\[
V = \frac{V_{\text{max}} [S]}{K_v + [S]}
\]

(Eq. 5)

Equation 3 is for competitive inhibition, whereas Equations 4 and 5 are for noncompetitive and uncompetitive inhibition, respectively.

**Equilibrium Binding Studies**—Quenching of the native tryptophan fluorescence of AOR upon substrate binding was used to measure dissociation constants \((K_v)\) of binding. Changes in tryptophan fluorescence were monitored using a PerkinElmer Life Sciences 50B luminescence spectrometer set at an excitation wavelength of 290 nm and an emission wavelength of 340 nm (slit widths: 5 and 10 nm, respectively).

**Steady-state kinetic and product inhibition studies**—Kinetic and Catalytic Mechanisms of AOR

Materials—trans-3-Nonen-2-one, trans-2-nonenal, and 2-nonanone were purchased from Cambridge Isotope Laboratories. The Developmental Therapeutics Program of the NCI, National Institutes of Health, supplied illudin M. All other chemicals and enzymes including glucose dehydrogenase from Cryptococcus unguillata and alcohol dehydrogenase from Thermoanaerobacter brockii were purchased from Sigma. Recombinant rat AOR was produced as described previously (1).

**Steady-state and Product Inhibition Studies**—Initial velocities of NADPH oxidation were measured at 25 °C using a Beckman DU70 spectrophotometer set to monitor absorbance at 340 nm. The assay buffer was optimized to contain 100 mM Tris (pH 7.2), 0.05% bovine serum albumin, 0.01% Triton X-100, and 150 mM NaCl. A 3-Nonen-2-one reduction was determined using 580 ng of AOR, 30–150 \( \mu \)M substrate dissolved in acetonitrile, and 24–48 \( \mu \)M NADPH in 1 ml of assay buffer. 2-Nonenal reduction was determined using 50–225 \( \mu \)M substrate and 4.5–6 \( \mu \)M NADPH. Data were fit to either of the following equations using GraFit 5.0.

\[
v = \frac{V_{\text{max}} A [B]}{K_A + A [B] + K_A [A] + [A] [B]}
\]

(Eq. 1)

\[
v = \frac{V_{\text{max}} A [B]}{K_A [A] + [A] [B]}
\]

(Eq. 2)

where \( K_A \) and \( K_B \) are the Michaelis constants for \( A \) and \( B \) and \( K_{\text{iso}} \) is the dissociation constant for \( A.\) Equation 1 is for a sequential mechanism with ternary complex formation, whereas Equation 2 is for a ping-pong bi bi kinetic mechanism. The F-test was used to evaluate best fits of the data.

**Product inhibition studies** using 3-nonen-2-one were carried out with similar assay conditions. When NADPH concentrations were varied, a constant, saturating 3-nonen-2-one concentration of 22.5 \( \mu \)M was used. Similarly, a NADPH concentration of 60 \( \mu \)M was employed when 3-nonen-2-one concentration was varied. In the 3-nonen-2-one versus 2-nonenal experiment, inhibitor concentrations of 0, 375, 845, and 750 \( \mu \)M were used, whereas the 3-nonen-2-one versus NADPH experiment employed inhibitor concentrations of 0, 50, 150, 250, and 400 \( \mu \)M. Similarly, in the NADPH versus 2-nonenone experiment, inhibitor concentrations of 0, 375, 750, and 1500 \( \mu \)M were used, whereas the NADPH versus NADPH experiment employed inhibitor concentrations of 0, 100, 150, and 250 \( \mu \)M. Progestosterone, a competitive (versus \( \alpha, \beta \)-unsaturated ketone) dead-end inhibitor of AOR was used at concentrations of 0, 50, 125, and 250 \( \mu \)M to determine its mode of inhibition versus NADPH. Data were fit to one the following equations using GraFit 5.0.

\[
v = \frac{V_{\text{max}} [S]}{K_v + [S]} + \frac{V_{\text{max}}}{K_v + [S]}
\]

(Eq. 3)

\[
v = \frac{V_{\text{max}} [S]}{K_v + [S]}
\]

(Eq. 4)

\[
v = \frac{V_{\text{max}} [S]}{K_v + [S]}
\]

(Eq. 5)

Equation 3 is for competitive inhibition, whereas Equations 4 and 5 are for noncompetitive and uncompetitive inhibition, respectively.

**Measurement of kinetic and solvent isotope effects**—Effects of deuterium-labeled NADPH on V and VM were measured using various concentrations of cofactors with a saturating (450 \( \mu \)M) concentration of 3-nonen-2-one. Deuterated assay buffer was prepared by dissolving 100 mM Tris, 0.05% bovine serum albumin, 0.01% Triton X-100, and 150 mM NaCl in D\(_2\)O and exchanging the solvent with fresh D\(_2\)O following evaporation with a SpeedVac. A pD of 7.2 was obtained using NaOD.

Solvent isotope effects were measured using a similar assay with 3-nonen-2-one concentrations ranging from 3.75 to 112.5 \( \mu \)M.
layer chromatography, dried with anhydrous sodium sulfate, and dis-
subsequently evaporated under vacuum. Thin layer chromatography
were then extracted three times with 50 ml of ethyl acetate, which was
These samples were incubated in the dark at 37 °C overnight. They
and undeuterated NADPH were used in separate incubations to pro-
slow in methanol-d₄ with tetramethylsilane for ¹H-NMR. NMR analyses
were completed on a Varian UNITY Plus 500-MHz NMR spectrom-
eter. Spectral assignments for the illudin M metabolite were determined previously (20). A two-dimensional NOESY ¹H NMR spec-
trum of the unlabeled metabolite was obtained using a 1-s mixing time.
Detection of Zn²⁺ in Enzyme Preparations—The His₆ tag of recombin-
ant AOR is an effective chelator of Zn²⁺ and thus required removal. This sequence was proteolytically cleaved from the protein using the
EKMax protease (Invitrogen) according to the manufacturer’s direc-
tions. AOR lacking the His₆ tag was then purified by ion exchange
chromatography using SP-fast flow resin (Amersham Biosciences) fol-
lowed by affinity chromatography using a High-Trap Blue column (Am-
ersham Biosciences). SDS-PAGE was used to assay fractions for purity. No His₆-tagged protein was detectable in the final preparation. Two protein samples were then dialyzed extensively (3 × 1 liter) in separate
plastic beakers against 0.5 × phosphate-buffered saline, made from 10× phosphate-buffered saline and distilled water (both supplied in plastic
bottles by Invitrogen). These samples were then assayed for activity
and protein content. They were then analyzed using an AAnalyst 600
atomic absorption spectrometer (PerkinElmer Life Sciences), which was
equipped with a Zn²⁺-specific lamp and calibrated with a solution of
ZnCl₂ concentrations as low as 10 µg of Zn²⁺/liter were used.

RESULTS

Steady-state Kinetic Studies—Initial velocity patterns were obtained when trans-3-nonen-2-one and trans-2-nonenal concentrations were varied at different fixed levels of NADPH. Double-reciprocal plots for both substrates are shown in Fig. 1 (A and B, respectively). When either substrate was used, lines intersected to the left of the ordinate, suggesting a sequential kinetic mechanism with ternary complex formation. Data were globally fit to Equation 2. Table I shows values measured for kinetic constants Kᵣ, Kᵢ, and Vₘₐₙₐ using 2-nonenal and 3-nonen-2-one as substrates. Due to sensitivity limitations of the spectrophotometric assay at low NADPH concentrations, the Kᵣ for 2-nonenal had to be obtained and verified through direct measurement. The Kᵣ for NADPH using 3-nonen-2-one as a substrate is ~10-fold higher than that calculated for 2-nonenal. The Kᵢ for NADPH using another α,β-unsaturated ketone, chalcone, was similar to that of 3-nonen-2-one and determined to be 31.8 ± 5.1 µM. Conversely, NADH was a poor cofactor; kinetic constants could not be measured, because enzymatic rates were extremely low (20-fold less) and linear at NADH concentrations up to 5.25 mM (data not shown). Clearly, NADPH is preferred over NADH. The reverse of 3-nonen-2-one reduction could not be detected when AOR was incubated with high concentrations of 2-nonenone and NADP⁺.

Poor solubility of the 2-nonenone metabolite, nonanal, pre-
cluded its use in product inhibition studies; rather, 3-nonen-2-
one and 2-nonanone were utilized (Table II). NADP$^+$ was found to exhibit competitive inhibition versus NADPH ($K_i = 79.9 \pm 7.2 \mu M$) and noncompetitive inhibition versus 3-nonen-2-one ($K_i = 140 \pm 16 \mu M$; $K_i = 421 \pm 82 \mu M$); these data were fit to Equations 3 and 4, respectively. 2-Nonanone was found to exhibit competitive inhibition versus 3-nonen-2-one ($K_i = 759 \pm 71 \mu M$) and noncompetitive inhibition versus NADPH ($K_i = 3510 \pm 570 \mu M$); these data were also fit to Equations 3 and 4, respectively. These data are consistent with either a rapid equilibrium random mechanism with two ternary abortive complexes formed or a Theorell-Chance ordered mechanism (21). Progesterone, a dead-end competitive inhibitor versus 3-nonen-2-one, was used to distinguish between the two. Progesterone demonstrated uncompetitive inhibition versus NADPH ($K_i = 759 \pm 71 \mu M$) and noncompetitive inhibition versus NADPH ($K_i = 3510 \pm 570 \mu M$); these data were also fit to Equations 3 and 4, respectively. These data are consistent with either a rapid equilibrium random mechanism with two ternary abortive complexes formed or a Theorell-Chance ordered mechanism (21).

Equilibrium Binding—Recombinant AOR contains five tryptophan residues, at least one of which is highly fluorescent. Quenching of this native fluorescence was utilized to measure the dissociation constants of NADPH and NADP$^+$ binding (Fig. 3). Nearly complete quenching ($\sim 88\%$) was observed with high concentrations of NADPH, whereas a more modest effect ($\sim 22\%$) was observed when using NADP$^+$. Data from titrations of the appropriate cofactors were fit to Equation 6 to calculate $K_d$ values of $8.8 \pm 0.8 \mu M$ for NADP$^+$ and $23.3 \pm 0.5 \mu M$ for NADPH. Similar experiments were attempted using 3-nonen-2-one and 2-nonanone; however, solubility issues prevented measurement of dissociation constants. No change in AOR fluorescence was observed with 2-nonanone concentrations up to $1 \text{mM}$. Whereas 3-nonen-2-one additions did slightly quench AOR fluorescence, saturation could not be reached at concentrations up to $1 \text{mM}$.

pH Profiles—The dependence of kinetic parameters of the NADPH-dependent activity of AOR on pH were determined over a range of $6.0$–$10.0$ (Fig. 4). The maximal velocity of 3-nonen-2-one reduction decreases at high pH as a functional group with a $pK_a$ of $9.2 \pm 0.07$ is deprotonated. Similar pH dependence was exhibited for $V/K_{\text{nonenone}}$ ($pK_a = 9.1 \pm 0.07$) and $V/K_{\text{NADPH}}$ ($pK_a = 9.3 \pm 0.04$). This functional group is probably acting as a general acid. No significant decrease in activity was detected at pH levels as low as $6.0$.

Deuterium and Solvent Kinetic Isotope Effects—Both [4S,2H]$^2$ and [4R,2H]$^2$NADPH were synthesized, purified as described under “Experimental Procedures,” and used to determine stereospecificity of hydride transfer. Initial velocities of cofactor oxidation were measured (Fig. 5). A saturating concentration (450 $\mu M$) of 3-nonen-2-one was used, whereas cofactor concen-
trations were varied from 15 to 200 μM. Rates were most diminished when [4R-2H]NADPH was used; thus, transfer of the 4R-H is probably catalyzed by AOR. Primary kinetic isotope effects of 1.16 ± 0.06 and 1.73 ± 0.22 were measured for V and VK, respectively, using [4R-2H]NADPH.

Solvent isotope effects of 4.30 ± 0.20 and 0.98 ± 0.8 were measured for V and VK, respectively (Fig. 6). Concomitant with the decrease in V, there was an approximate 4-fold decrease in K_m. This change is probably due to a conformational change in the enzyme active site caused by the replacement of a structural water with deuterium oxide. Thus, whereas the solvent isotope effects are large, they may not accurately represent the kinetics of chemical events.

Regio- and Stereoselectivity of Hydride Transfer—Illudin M was used to determine the stereosepecificity of hydride transfer both from NADPH and to an α,β-unsaturated ketone (Fig. 7). Upon reduction of the 8,9-double bond of illudin M, the ketone undergoes a tautomeric rearrangement to form an unstable cyclohexadiene intermediate. The cyclopropyl group of this intermediate is very susceptible to attack by nucleophiles, particularly hydroxide and chloride ions (20). After the opening of the cyclopropyl ring and expulsion of water, an aromatic product is formed (Fig. 7A). Presumably, because the α,β-double bond of illudin M is situated on a cyclopentyl ring that contains methyl and hydroxyl substituents, protons of the 8-carbon of the metabolite (the two doublets at δ 2.49–2.53 and 2.74–2.78) are not equivalent and resolve discretely in 1H NMR spectra (Fig. 7B). [4R-2H]NADPH and [4R-1H]NADPH were added to separate incubations of AOR and illudin M in chloride-free potassium phosphate buffer. Metabolites were then purified and subjected to 1H NMR spectroscopic analyses. Nonspecific proton assignments were completed previously (20). Upon comparison of the spectra, it is apparent that the doublet at δ 2.74–2.78 is lost when [4R-2H]NADPH cofactor is used (Fig. 7C). Additionally, consistent with the inability of deuterium to couple with neighboring protons, the doublet at δ 2.49–2.53 becomes a singlet.

Two-dimensional NOESY was employed to stereospecifically assign the nonequivalent 8-protons, labeled H_1 and H_2 in Fig. 8. The proton of the 6-carbon (δ 4.47) interacts strongly with protons of the 13- and 14-methyl groups and weakly with those at the 15-position. The 14-methyl protons in turn interact with the 8-carbon proton at δ 2.49–2.53. The protons of the 15-methyl group interact with the 8-carbon proton at δ 2.74–2.78. A schematic of these interactions is drawn in Fig. 8. One would expect a different two-dimensional spectra if the cyclopentyl group were in the opposite “pucker” position. It is clear from the above interpretation that protons H_1 and H_2 can be assigned to the δ 2.49–2.53 and 2.74–2.78 doublets, respectively. These data demonstrate that AOR catalyzes the transfer of the pro-R hydride from NADPH to the β-carbon of the α,β-double bond. Further, if the α,β-unsaturated carbonyl is situated with the oxygen to the right of the carbonyl carbon, the hydride is transferred from underneath and will become the pro-R proton.

Assays for Zn^{2+} Presence—Since many MDR family members utilize Zn^{2+} in catalytic and/or structural roles (23), it was of interest to determine whether this cation is required for NADPH-dependent AOR activity. The addition of the chelator 1,10-phenanthroline had no effect on the rate of NADPH oxidation, whereas the addition of 250 μM Zn^{2+} inhibited AOR activity more than 2-fold (data not shown). Further, atomic absorption spectroscopy was used to verify that active AOR preparations did not contain stoichiometric amounts of Zn^{2+}. AOR concentrations of 1.6–1.7 μM contained less than 4 × 10^{-6} μM Zn^{2+}.

**DISCUSSION**

AOR was first isolated in a search of the porcine kidney for an enzyme with NADP^+ dependent LTB4, 12-hydroxydehydrogenase activity (10). This activity serves to virtually inactivate this potent neutrophil chemoattractant and stimulator of degranulation. It was later found that this enzyme is coordinately regulated with several antioxidative and conjugative phase 2 enzymes in the rat liver (2) and has the NAD(P)H-dependent ability to hydroxenate the 13,14-double bond of the 15-oxoprostaglandins (6). AOR was also found to catalyze the reduction of an array of smaller α,β-unsaturated carboxyls, including cyto-
This experiment is representative of two separate preparations of D2O-to mammalian homology to characterized MDRs. Due to significant homology, G[2H]NADPH ([2H]) and [4-17274-2H]NADPH, predicted to catalyze one-electron reductions of quinones. However, a primary kinetic isotope effect of 0.98 ± 0.8. This experiment is representative of two separate preparations of D2O-exchanged buffer and assays.

Toxic products of lipid peroxidation, at higher rates. Lipid peroxidation occurs under conditions of oxidative stress and involves the reaction of radical oxygen species with polyunsaturated fatty acids to produce a variety of electrophilic α,β-unsaturated aldehydes. Because hydrogenation of the α,β-double bonds of these molecules translates to dramatic reductions in cytotoxicity, the protective, antioxidative role of the oxidoreductase activity of AOR was elucidated (1).

AOR has recently been classified (24) as belonging to the MDR superfamily (Fig. 9). This classification is largely based on its LTB4 12-hydroxysteroid dehydrogenase activity and sequence homology to characterized MDRs. Due to significant homology to mammalian ζ-crystallin and E. coli quinone reductase, AOR was further classified as an oxidoreductase (11). Characteristics of this subfamily include a preference for NADP(H) over NAD(H) and lack of Zn²⁺ dependence. E. coli quinone reductase (25) and guinea pig ζ-crystallin (26) are only known to catalyze one-electron reductions of quinones. However, a ζ-crystallin homolog isolated from A. thaliana (P1-ζ-crystallin) is also capable of two-electron reductions of azocarbonyls (15), such as diame and, several α,β-unsaturated aldehydes and ketones (16), including 4HNE. Interestingly, this enzyme is up-regulated by oxygen stressors and is postulated to play an antioxidative role similar to AOR (16). P1-ζ-crystallin and AOR share 58% sequence homology; however, AOR appears to be more closely related to a set of putative microbial oxidoreductases designated in the COG data base as COG2130 and represented in Fig. 9 by YNCB from E. coli and a putative oxidoreductase from R. solanacearum.

Whereas the enzymology of LADHs has been extensively characterized (23), little is known of the catalytic mechanisms of AOR and the other MDR reductases. Multiple sequence alignments have allowed for predictions of active site size and composition, Zn²⁺ requirements, cofactor preference, etc.; however, they have remained relatively unproven (11). With their validation and the uniqueness of its dual activities in mind, we undertook an investigation of the catalytic mechanism of the oxidoreductase activity of AOR.

Steady state and product inhibition kinetic studies were employed to determine the kinetic mechanism of AOR. Using both α,β-unsaturated aldehyde and ketone substrates, double-reciprocal plots exhibited intersecting patterns suggesting ternary complex formation (Fig. 1). In product inhibition studies, NADP⁺ demonstrated noncompetitive inhibition versus 3-nonen-2-one and competitive inhibition versus NADPH (Table II). Similarly, 2-nonanone exhibited competitive inhibition versus 3-nonen-2-one and noncompetitive inhibition versus NADPH. This pattern is representative of either an ordered Theorell-Chance or random equilibrium bi bi mechanism with abortive complex formation (21). An ordered mechanism was verified using a dead end competitive inhibitor of α,β-unsaturated ketones, progesterone (Fig. 2).

Thus, the NADPH-dependent activity of AOR best conforms to an ordered Theorell-Chance kinetic mechanism (Fig. 10A). This mechanism involves ordered binding of substrates followed by rapid catalysis and dissociation of the first product followed by slower dissociation of the second. The rapidity of both chemical interconversion and first product dissociation are the characteristics that distinguish it from an ordered bi bi mechanism. In this study, NADPH was found to bind before 3-nonen-2-one, which is then rapidly reduced to 2-nonanone and leaves before NADP⁺. This mechanism is supported by relatively small primary kinetic isotope effects, which demonstrate that hydride transfer is not rate-limiting (Fig. 5). Further, a high Kᵣ value for 2-nonanone in product inhibition studies (Table II) and an unmeasurable Kᵣ in equilibrium binding studies hint for a low affinty of this product for AOR.
Horse LADH demonstrates an ordered bi bi kinetic mechanism with cofactor binding first (27, 28). It is possible that other AOR substrates, with greater product affinities, may be metabolized according to this mechanism. Dissociation of NADP$^+$ is a probable candidate for the rate-limiting step due to relatively low $K_i$ (Table II) and $K_f$ (Fig. 3) values.

Cofactor preference was also investigated in this study. Whereas MDR dehydrogenases display equal or greater preference for NAD(H), MDR reductases greatly prefer NADPH (11). In accord with its classification as a reductase, AOR displays a vast preference for NADPH. Interestingly, $K_m$ values for NADPH differed by nearly an order of magnitude depending on the identity of the unsaturated substrate (Table I). Whereas unsaturated ketones, 3-nonen-2-one, and chalcone, demonstrate $K_m$ values in the 20–30 μM range, the cofactor $K_m$ when using the aldehyde 2-nonenal substrate, was 4 μM. It is possible that the aldehyde moiety is acting through an allostERIC site. The $K_J$ for NADPH, in the absence of additional substrate, was found to be 23.3 μM and thus in accord with $K_m$ values measured for ketones.

The effect of pH on AOR activity was also investigated. Values of $pK_a$ for $V_{max}$, $V/K_{homo}$, and $V/K_{NADPH}$ were determined to be 9.2, 9.1, and 9.3, respectively (Fig. 4). AOR displayed no significant loss of activity at pH 6.0. A functional group with a $pK_a$ of −9.2 is probably acting as a general acid in the catalytic process.

The regioselectivity and stereospecificity of hydride transfer from NADPH to an α,β-unsaturated ketone was also investigated. NADPH labeled with deuterium at the pro-R position exhibited greater kinetic isotope effects than [4S-2H]NADPH and was therefore used in substrate labeling experiments (Fig. 7). The mycotoxin illudin M was chosen because protons at the 8-C position of its metabolite are not equivalent in 1H NMR spectra (Fig. 7B) and can be used to determine the stereospecificity of hydride transfer to the substrate. A 1H NMR spectrum of deuterium-labeled illudin M metabolite verified that the pro-R hydrogen of NADPH is transferred to the β-carbon of an α,β-unsaturated ketone by AOR. Horse LADH also utilizes the pro-R hydrogen (29). The β-carbon is the most electropositive of the α,β-unsaturated carbonyl and is the site of nucleophilic attack in Michael addition chemical reactions. A two-dimensional NOESY NMR spectrum of the unlabeled metabolite was obtained to stereospecifically assign protons of the 8-C position (Fig. 8). The deuterium label was determined to have been transferred to the R-position of the metabolite, which translates to attack from below if the α,β-unsaturated ketone is conceptualized as lying on its side with the oxygen to the right of the carbonyl carbon.

Most MDR dehydrogenases are known to require Zn$^{2+}$ for structural integrity and/or catalysis (11). In the later case, the electropositive metal is used to polarize carbonyls and facilitate formation of alkoxide transition states. However, multiple sequence alignments have predicted that MDR members classified as oxidoreductases lack the common Zn$^{2+}$-binding loop exemplified by horse LADH (Fig. 9). The crystal structure of E. coli quinone reductase demonstrates that this oxidoreductase may instead use a proximal cluster of positively charged amino acids (30, 31). Using atomic absorption spectroscopy, active preparations of AOR were found to be devoid of Zn$^{2+}$. Further evidence for Zn$^{2+}$ independence came from Zn$^{2+}$ chelation and supplementation studies.

The finding that NADP$^+$ competitively inhibits NADPH demonstrates that they probably bind to the same site on AOR. This supports the notion that both hydroxyl oxidation and carbon-carbon double bond reduction occur in the same active site. LTB$_4$ was found to inhibit 3-nonen-2-one reduction, but...
concentrations high enough to determine its mode of inhibition were not possible. If this assumption is correct and $\alpha,\beta$-unsaturated alcohols and carbonyls bind in the same orientation, AOR is capable of transferring hydride ion over distances spanning two bond lengths. Alcohol dehydrogenases normally abstract alcohol carbon hydrides, whereas AOR was shown to transfer hydride to the $\beta$-carbon (Fig. 7). It was found that $\alpha,\beta$-unsaturated ketones constrained to a transoid configuration across their carbonyl carbon-$\alpha$ carbon single bond, as seen in 2-cyclohexenone, are not metabolized by AOR (1). It is possible that AOR requires either flexibility about this bond or the carbonyl oxygen and $\beta$-carbon to be in close proximity.

Insights into the shape and composition of the active site have come from homology modeling and substrate specificity studies. Nordling et al. (11) have modeled human AOR based on the E. coli quinone reductase crystal structure. They have predicted the active site to contain sparse charged residues and to be deep, narrow, hydrophobic, and polarized. We have found that AOR is able to reduce a variety of $\alpha,\beta$-unsaturated aldehydes and ketones. In agreement with active site characteristics, the more hydrophobic molecules were generally better substrates, with substituents of aliphatic straight chains and

**Fig. 8.** Two-dimensional NOESY NMR spectrum of illudin M metabolite. Two-dimensional nuclear Overhauser effect spectroscopy was used to stereospecifically assign the nonequivalent C-8 position protons. The doublet at $\delta$ 2.74–2.78 is determined to represent the C-8 proton at the R stereoposition.

**Fig. 9.** Representative neighbor-joining tree of MDR family members. This tree was constructed using ClustalW with a PAM matrix. A nearly complete collection of characterized MDR reductases is boxed. Three well-characterized MDR dehydrogenases are located outside of the box: CAD, cinnamyl alcohol dehydrogenase; YADH, yeast alcohol dehydrogenase; QOR, quinone reductase; YNCB, putative prokaryotic oxidoreductase; LTB4DH, leukotriene B$_4$ 12-hydroxydehydrogenase.
phenyl groups equally well tolerated (1). Any branching at the α or β positions was not well tolerated. Ketones were better substrates than aldehydes, and more hydrophobic substituents of the carbonyl carbon translated to dramatic decreases in $K_m$. AOR could have evolved from a primitive allylic alcohol dehydrogenase. It is capable of catalyzing the oxidation of an α,β-unsaturated alcohol but, unlike allyl alcohol dehydrogenase (Nicotiana tabacum), is unable to catalyze the reverse reaction (32). Instead, AOR has evolved the ability to transfer the pro-R hydride of NADPH to what becomes the R-position hydrogen of the β-carbon.

Partial characterization of the catalytic mechanism of AOR has justified its classification as an MDR oxidoreductase. Predictions of cofactor preference and lack of Zn$^{2+}$ requirements have been verified, whereas kinetic and stereospecificity characteristics have proven consistent with distantly related LADH. In general, the MDR reductases are a poorly characterized class of enzymes. The majority of its members are putative gene products identified by genome sequencing. The results of this study may help to guide their characterization and in the process define the molecular evolution of AOR.
