Formamides Mimic Aldehydes and Inhibit Liver Alcohol Dehydrogenases and Ethanol Metabolism*

Received for publication, May 23, 2003

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Title Footnotes:

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The atomic coordinates and structure factors (code 1P1R) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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Formamides are unreactive analogues of the aldehyde substrates of alcohol dehydrogenases and are useful for structure-function studies and for specific inhibition of alcohol metabolism. They bind to the enzyme-NADH complex and are uncompetitive inhibitors against varied concentrations of alcohol. Fourteen new branched chain and chiral formamides were prepared and tested as inhibitors of purified Class I liver alcohol dehydrogenases: horse (EqADH E), human (HsADH1C*2), and mouse (MmADH1). In general, larger, substituted formamides, such as N-1-ethylheptylformamide, are better inhibitors of HsADH1C*2 and MmADH1 than of EqADH, reflecting a few differences in amino acid residues that change the sizes of the active sites. In contrast, the linear, alkyl (n-propyl and n-butyl) formamides are better inhibitors of EqADH and MmADH1 than of HsADH1C*2, probably because water disrupts van der Waals interactions. These enzymes are also inhibited strongly by sulfoxides and 4-substituted pyrazoles. The structure of EqADH complexed with NADH and (R)-N-1-methylhexylformamide was determined by X-ray crystallography at 1.6 Å resolution. The structure resembles the expected Michaelis complex with NADH and aldehyde, and shows for the first time that the reduced nicotinamide ring of NADH is puckered, as predicted for the transition state for hydride transfer. Metabolism of ethanol in mice was inhibited by several formamides. The data were fitted with kinetic simulation to a mechanism that describes the non-linear progress curves and yields estimates of the in vivo inhibition constants and the rate constants for elimination of inhibitors. Some small formamides, such as N-isopropylformamide, may be useful inhibitors in vivo.
Vertebrate alcohol dehydrogenases (E. C. 1.1.1.1, ADH<sup>1</sup>) are involved in the metabolism of relatively non-polar alcohols and aldehydes (1). The five different human enzymes have broad substrate specificity, oxidizing primary and secondary alcohols and reducing their corresponding carbonyl compounds. Specific inhibitors of these dehydrogenases would be useful for defining substrate specificity, for studying the physiological roles of the enzymes and for preventing the oxidation of methanol and ethylene glycol to toxic products (2-6).

Formamides and sulfoxides are analogues of the carbonyl substrates, are bound preferentially to the enzyme-NADH complex, and are potent uncompetitive inhibitors against varied concentrations of alcohol (7-15). Uncompetitive inhibitors could be especially useful for controlling alcohol metabolism since they are effective even when the concentrations of alcohol are saturating. Previous studies identified some potent uncompetitive inhibitors for human (14, 15), horse (10, 11, 14, 15), monkey (11), and rat (10, 11) liver alcohol dehydrogenases. We now extend the studies with 14 new branched-chain and chiral formamides in order to explore active site topologies and to make more effective inhibitors for the mouse (M<sub>m</sub>ADH1) and human (H<sub>s</sub>ADH1C<sup>*2</sup>) enzymes. For comparison, we also studied the potency of five sulfoxides and two 4-substituted pyrazoles with purified M<sub>m</sub>ADH1.

We also studied the inhibition by five compounds of ethanol metabolism in mice as a model animal. We used numerically integrated rate equations that describe the non-linear progress curves and provide good estimates of inhibition constants <i>in vivo</i>. The results provide information for optimizing the sizes of inhibitors for maximal efficacy.

We determined by X-ray crystallography the structure of the complex of horse liver ADH with NADH and a potent, chiral formamide inhibitor, which binds as an analogue of an aldehyde. The structure shows, for the first time in a ternary complex of a dehydrogenase with
NADH, that the reduced nicotinamide ring is puckered, as expected for the transition state for hydrogen transfer. The structure also provides a basis for interpreting the structure-function relationships of the inhibitors.

EXPERIMENTAL PROCEDURES

Materials–Native crystalline horse liver alcohol dehydrogenase (EE isoenzyme), LiNAD$^+$ and Na$_2$NADH were purchased from Roche Molecular Biochemicals. The plasmid for the expression of $H$sADH1C*2 was obtained from Dr. Jan-Olov Höög (Karolinska Institutet, Stockholm, Sweden) and that of $M$mADH1 from Dr. Gregg Duester (Burnham Institute, La Jolla, CA). Amines and other reagents were obtained from Lancaster and Aldrich Chemical Co. Some formamides and sulfoxides were prepared previously (11, 14).

Synthesis of Formamides–The formamides were generally prepared (unless stated otherwise) by refluxing the amine with 88% HCOOH, removing excess HCOOH and H$_2$O under reduced pressure, and distilling the product. $N$-1-Cyclohexylethyl and $N$-1-(4-methylphenyl)-ethyl formamides were solids and were recrystallized from ethyl acetate/hexane or dichloromethane. Final yields were 50-70%. Compounds were characterized using a Varian 500 MHz or a Bruker AC 300 NMR spectrometer. Multiple resonances are due to the cis-trans ($Z$, $E$) isomerism of the formamide group. The major geometrical isomer was cis (~70-80%). Mass spectra (MS or high resolution, HRMS) were obtained using a Micromass Autospec M spectrometer. The structure of each compound was confirmed by three different analytical methods.

$N$-1,2-Dimethylpropylformamide–$^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ = 8.15 (0.8H, s, CHO), 8.04 (0.2H, d, $J$ = 12 Hz, CHO), 6.16 and 5.98 (1H, 2br s, NH), 3.95 and 3.3 (1H, 2m, CH), 1.71 (1H, m, CH), 1.2 and 1.1 (3H, 2d, $J$ = 6.7 Hz, CH$_3$), 0.93 (3H, d, J = 6.6 Hz, CH$_3$), 0.92 (3H, d, J = 6.6 Hz, CH$_3$); $^{13}$C NMR (CDCl$_3$, 75.5 MHz): $\delta$ = 164.2 ($E$-CHO), 160.7 ($Z$-CHO), 53.7 ($E$-N-
\[
\begin{align*}
\text{CH)} & \text{, 48.8 (Z-N-CH), 33.8 (E-CH-CH), 32.8 (Z-CH-CH), 18.4, 17.5; Anal. (C}_6\text{H}_{13}\text{NO}^{1/12}\text{H}_2\text{O}) \\
\text{C, H, N.}
\end{align*}
\]

\textit{N-Isopentylformamide}–\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz): \(\delta = 8.1\) (1H, s, CHO), 6.5 (1H, br s, NH), 3.3-3.2 (2H, 2m, CH\textsubscript{2}), 1.6 (1H, m, CH), 1.4 (2H, m, CH\textsubscript{2}), 0.88 (6H, d, J = 5.1 Hz, CH\textsubscript{3}); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75.5 MHz): \(\delta = 165.0\) (E-CHO), 161.8 (Z-CHO), 40.2 (E-N-CH), 38.3 (Z-N-CH), 36.6, 25.8, 22.4, 22.3; Anal. (C\textsubscript{6}H\textsubscript{13}NO\textsubscript{1/12}H\textsubscript{2}O) C, H, N.

\textit{N-1,4-Dimethylpentylformamide}–\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz): \(\delta = 8.13\) (0.8H, s, CHO), 8.06 (0.2H, d, J = 12 Hz, CHO), 5.73 and 5.55 (1H, 2br s, NH), 4.04 and 3.45 (1H, 2m, CH), 1.6-1.4 (3H, m, CH and CH\textsubscript{2}), 1.24-1.2 (2H, m, CH\textsubscript{2}), 1.16 (3H, d, J = 6.9 Hz, CH\textsubscript{3}), 0.88 (6H, d, J = 6.6 Hz, CH\textsubscript{3}); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75.5 MHz): \(\delta = 163.9\) (E-CHO), 160.6 (Z-CHO), 48.9 (E-N-CH), 44.3 (Z-N-CH), 35.0, 34.6, 27.8, 22.6, 22.5, 20.9; Anal. (C\textsubscript{8}H\textsubscript{17}NO\textsubscript{1/8}H\textsubscript{2}O): C, H, N.

\textit{(R)-N-1-Methylhexylformamide}–\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 300 MHz): \(\delta = 8.10\) (0.8H, s, CHO), 8.06 (0.2H, d, J = 12 Hz, CHO), 5.8 and 5.68 (1H, 2br s, NH), 4.07 and 3.47 (1H, 2m, CH), 1.50-1.20 (8H, m, CH\textsubscript{2}), 1.17 (3H, d, J = 6.6 Hz, CH\textsubscript{3}), 0.87 (3H, m, CH\textsubscript{3}); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75.5 MHz): \(\delta = 164.1\) (E-CHO), 160.8 (Z-CHO), 48.7 (E-N-CH), 44.3 (Z-N-CH), 37.9 (E-N-CH-CH\textsubscript{2}), 36.9 (Z-N-CH-CH\textsubscript{2}), 31.8, 25.8, 22.7, 21.0, 14.1; MS (EI) \textit{m/z} (% relative intensity) 144 [11, (M+H\textsuperscript{+})], 143 (1, M\textsuperscript{+}) 128 (48, M\textsuperscript{+}-CH\textsubscript{3}), 114 (34, M\textsuperscript{+}-C\textsubscript{2}H\textsubscript{5}), 72 (100, M\textsuperscript{+}-C\textsubscript{5}H\textsubscript{11}); HRMS calculated for C\textsubscript{8}H\textsubscript{17}NO 143.1311, found 143.1319.

\textit{(S)-N-1-Methylhexylformamide}–\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 500 MHz): \(\delta = 8.18\) (0.8H, s, CHO), 8.11 (0.2H, d, J = 8.6 Hz, CHO), 6.1 and 5.6 (1H, 2br s, NH), 4.14 and 3.55 (1H, 2m, CH), 1.5-1.2 (8H, m, CH\textsubscript{2}), 1.17 (3H, d, J = 6.5 Hz, CH\textsubscript{3}), 0.88 (3H, m, CH\textsubscript{3}); \textsuperscript{13}C NMR (CDCl\textsubscript{3}, 75.5 MHz): \(\delta = 164.1\) (E-CHO), 160.9 (Z-CHO), 48.8 (E-NH-CH), 44.3 (Z-NH-CH), 37.8 (E-N-CH-CH\textsubscript{2}), 36.8 (Z-N-CH-CH\textsubscript{2}), 31.7, 25.8, 22.7, 21.0, 14.1; MS (EI) \textit{m/z} (% relative intensity) 144 [6,
(M+H)⁺], 143 (1, M⁺), 128 (48, M⁺-CH₃), 114 (34, M⁺-C₂H₅), 72 (100, M⁺-C₅H₁₁); HRMS calculated for C₈H₁₇NO 143.1310, found 143.1308.

**N-1-Methylhexylformamide**—¹H NMR (CDCl₃, 300 MHz): δ = 8.04 (0.8H, s, CHO), 7.99 (0.2H, d, J = 12 Hz, CHO), 5.98 and 5.72 (1H, 2br s, NH), 3.98 and 3.40 (1H, 2m, CH), 1.38-1.14 (8H, m, CH₂), 1.08 (3H, d, J = 6.6 Hz, CH₃), 0.81 (3H, m, CH₃); ¹³C NMR (CDCl₃, 75.5 MHz): δ = 164.0 (E-CHO), 160.8 (Z-CHO), 48.7 (E-N-CH), 44.4 (Z-N-CH), 38.0 (E-N-CH-C₂H₂), 37.0 (Z-N-CH-C₂H₂), 31.9, 25.8, 22.8, 21.2, 14.2; Anal. (C₈H₁₇NO·1/₁₂H₂O) C, H, N.

**N-1,5-Dimethylhexylformamide**—¹H NMR (CDCl₃, 500 MHz): δ = 8.08 (0.8H, s, CHO), 8.04 (0.2H, d, J = 12 Hz, CHO), 6.22 and 6.06 (1H, 2br s, NH), 3.99 and 3.43 (1H, 2m, CH), 1.52-1.05 (m, 7H, CH and CH₂), 1.15 (3H, d, J = 6.8 Hz, CH₃), 0.86 (6H, d, J = 6.2 Hz, CH₃); ¹³C NMR (CDCl₃, 75.5 MHz): δ = 163.8 (E-CHO), 160.7 (Z-CHO), 48.5 (E-NH-CH), 44.0 (Z-NH-CH), 38.6, 36.8, 27.7, 23.6, 22.4, 22.4, 20.7; Anal. (C₉H₁₇NO·1/₁₂H₂O) C, H, N.

**N-1-Ethylpropylformamide**—¹H NMR (CDCl₃, 500 MHz): δ = 8.31 (0.6H, s, CHO), 7.97 (0.4H, d, J = 12 Hz, CHO), 6.59 and 5.94 (1H, 2br s, NH), 3.83 and 3.08 (1H, 2m, CH), 1.71-1.34 (4H, m, CH₂), 0.97 and 0.89 (6H, 2t, J = 7.5 Hz, CH₃); ¹³C NMR (CDCl₃, 75.5 MHz): δ = 167.3 (Z-CHO), 165.2 (E-CHO) 56.8 (E-N-CH), 54.6 (Z-N-CH), 28.7 (E-N-CH-C₂H₂), 27.5 (Z-N-CH-C₂H₂), 25.3, 10.4, 9.7; Anal. (C₆H₁₃NO·H₂O.1/₄HCOOH) C, H, N.

**N-1-Ethylhexylformamide**—As for the first stage of the Leukart reaction, ammonium formate and 3-octanone (4:1 molar ratio) were heated (140–150 °C) until the mixture became homogeneous. Water and some 3-octanone distilled over. The ketone was returned to the reaction vessel, and the mixture was heated at 155–160 °C for 4 hr. The cooled reaction mixture was washed twice with water, and the product was distilled. ¹H NMR (CDCl₃, 300 MHz): δ = 8.19 (0.7H, s, CHO), 8.01 (0.3H, d, J = 9 Hz, CHO), 6.14 and 5.95 (1H, 2br s, NH), 3.91 and
3.12 (1H, 2m, CH), 1.62-1.20 (10H, m, CH₂), 0.95-0.85 (6H, m, CH₃); $^{13}$C NMR (CDCl₃, 75.5 MHz): $\delta = 164.5$ ($E$-CHO), 161.2 ($Z$-CHO), 54.8 ($E$-N-CH), 49.7 ($Z$-N-CH), 35.8 ($E$-N-CH-CH₂), 34.7 ($Z$-N-CH-CH₂), 31.9, 28.0, 25.7, 22.7, 14.1, 10.3; MS (EI) m/z (% relative intensity) 158 [2, (M+H)+], 128 (86, M+-C₂H₅), 86 (100, M+-C₅H₁₁); HRMS calculated for C₇H₁₄NO (M+-C₂H₅) 128.1075, found 128.1078.

$N$-1-Ethyleptylformamide—$^1$H NMR (CDCl₃, 500 MHz): $\delta = 8.23$ (0.5H, s, CHO), 7.99 (0.5H, d, J = 12 Hz, CHO), 6.96 and 5.55 (1H, 2br s, NH), 3.95 and 3.2 (1H, 2m, CH), 1.67-1.21 (12H, m, CH₂), 0.96-0.87 (6H, m, CH₃); $^{13}$C NMR (CDCl₃, 75.5 MHz): $\delta = 165.6$ ($E$-CHO), 161.9 ($Z$-CHO), 55.5 ($E$-N-CH), 50.1 ($Z$-N-CH), 35.6 ($E$-N-CH-CH₂), 34.7 ($Z$-N-CH-CH₂), 31.9, 29.3, 27.9, 26.0, 22.7, 14.2, 10.3; Anal. (C₁₀H₂₁NO·1/2H₂O·1/₃HCOOH) C, H, N.

$N$-3-Methylcyclohexylformamide—$^1$H NMR (CDCl₃, 300 MHz): $\delta = 8.15$ and 8.09 (1H, 2s, CHO), 5.88 and 5.64 (1H, 2br s, NH), 3.85 and 3.30 (1H, 2m, CH), 1.98-0.76 (9H, m, ring CH₂), 0.91 (3H, d, J = 6.6 Hz, CH₃); $^{13}$C NMR (CDCl₃, 75.5 MHz): $\delta = 163.6$ ($E$-CHO), 160.4 ($Z$-CHO), 51.4 ($E$-N-CH), 47.5 ($Z$-N-CH), 43.4 ($E$-N-CH-CH₂), 41.9 ($Z$-N-CH-CH₂), 34.2, 32.8, 31.8, 24.8, 22.4; Anal. (C₈H₁₅NO·1/₃H₂O) C, H, N.

$N$-2-Methylcyclohexylformamide—$^1$H NMR (CDCl₃, 300 MHz): $\delta = 8.17$ (0.7H, dd, J = 7.2 Hz and 1.2 Hz, CHO), 8.04 (0.3H, dd, J = 12 Hz and 3.9 Hz, CHO), 6.2 and 6.1 (1H, 2br s, NH), 3.54 and 2.79 (1H, 2m, CH), 2.0-1.0 (9H, m, ring CH₂), 0.94 (3H, d, J = 6.3 Hz, CH₃); $^{13}$C NMR (CDCl₃, 75.5 MHz): $\delta = 164.1$ ($E$-CHO), 160.8 ($Z$-CHO), 57.8 ($E$-N-CH), 52.9 ($Z$-N-CH), 38.3 ($E$-N-CH-CH₂), 38.1 ($Z$-N-CH-CH₂), 34.2, 33.5, 25.6, 25.4, 19.1; Anal. (C₈H₁₅NO·1/₃HCOOH·H₂O) C, H, N.

(R)-N-1-Cyclohexylethylformamide—$^1$H NMR (CDCl₃, 500 MHz): $\delta = 8.17$ (0.7H, s, CHO), 8.05 (0.3H, d, J = 12 Hz, CHO), 5.95 and 5.73 (1H, 2br s, NH), 3.93 and 3.25 (1H, 2m, CH),
1.79-1.61 (5H, m, CH and CH$_2$), 1.33-0.82 (6H, m, CH$_2$), 1.10 (3H, d, J = 6.5 Hz, CH$_3$); $^{13}$C NMR (CDCl$_3$, 75.5 MHz): δ = 164.3 (E-CHO), 160.9 (Z-CHO), 53.3 (E-N-CH), 48.3 (Z-N-CH), 43.8 (E-N-CH-CH$_2$), 43.0 (Z-N-CH-CH$_2$), 29.2, 29.1, 26.5, 26.3, 26.2, 18.0; Anal. (C$_9$H$_{17}$NO) C, H, N; m.p. 48-49 °C.

(R)-N-1-(4-Methylphenyl)-ethylformamide–$^1$H NMR (CDCl$_3$, 300 MHz): δ = 8.14 (1H, s, CHO), 7.2 (4H, 2d, J = 9 Hz, aromatic protons), 5.92 (1H, br s, NH), 5.17 and 4. 65 (1H, 2m, CH), 2.33 (3H, s, CH$_3$), 1.49 (3H, d, J = 6.9 Hz, CH$_3$); $^{13}$C NMR (CDCl$_3$, 75.5 MHz): δ = 164.4 (E-CHO), 160.6 (Z-CHO), 129.5 (3C), 126.2 (3C), 51.7 (E-N-CH), 47.5 (Z-N-CH), 22.0, 21.2; Anal. (C$_{10}$H$_{13}$NO) C, H, N; m.p. 64 °C.

Protein Purification–Human alcohol dehydrogenase 1C*2 was prepared essentially as described (16). Mouse ADH1 was purified using the following procedure, which gave better yields than the general procedure suggested by Qiagen. Mouse ADH1 was expressed in *Escherichia coli* M15 (pREP4) from the full-length cDNA linked to a 6x His tag in the pQE-30 plasmid (Qiagen, Valencia, CA). The *E. coli* cells were grown at 37 °C on plates and in starter cultures with LB media containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin and used to inoculate 9 L cultures of LB without antibiotics. At an optical density of 0.6-0.8 at 600 nm, expression was induced with 0.5 mM isopropyl thiogalactoside. Cells were allowed to grow at 30 °C for 16 hr, collected by centrifugation and stored at –20 °C overnight. Cell paste was thawed and suspended in 3 ml of 50 mM Tris-HCl, 0.25 mM EDTA, pH 8.0, per g of cell paste. Subsequent steps were at 0–4 °C. Lysozyme (1 mg/ml, total volume) was added, and the suspension was stirred for 30 min. The cells were sonicated for 12 cycles of 30 s each with intermittent cooling. Protamine sulfate was added to a final concentration of 0.2%, and the mixture was centrifuged after stirring for 30 min. The supernatant was applied to a DEAE
Sepharose Fast Flow column (100 ml), and the enzyme was eluted with 50 mM Tris-HCl buffer, pH 8.0. The fractions with ADH activity were concentrated with an Amicon concentrator with a YM30 membrane under 40 psi N₂ while the buffer was changed to 50 mM sodium phosphate buffer containing 300 mM sodium chloride and 10 mM imidazole, pH 8.0. The enzyme was loaded onto a 15 ml nickel nitrilotriacetate-affinity column, which was washed with 50 mM sodium phosphate, 300 mM sodium chloride and 20 mM imidazole buffer, pH 8.0, until the effluent had negligible protein. The column was developed with a 200 ml linear gradient from 20 to 250 mM imidazole. Fractions with ADH activity were concentrated with an Amicon concentrator with a YM30 membrane under 40 psi N₂ while the buffer was changed to 50 mM Tris-HCl and 0.25 mM EDTA buffer, pH 8.0, in order to remove imidazole. The enzyme was again loaded onto a DEAE column (15 ml), eluted with the 50 mM Tris-HCl buffer, and concentrated and stored at 4 ºC.

About 100 mg of protein was obtained from 9 L of culture. Electrophoretic analysis using polyacrylamide gel with sodium dodecyl sulfate showed a single band at the position expected for an ADH subunit (40 kDa). The ratio of tyrosine to tryptophan predicted from the sequence (3:2) was confirmed with an UV-absorption spectrum of the protein in 0.1 M NaOH (17). The absorption at 280 for a 1 mg/ml solution was estimated to be 0.37 at neutral pH from the spectrum in alkali. The turnover number was determined to be 0.66 s⁻¹ and 1.07 s⁻¹ based on a standard assay (18) at 25 ºC and 37 ºC, respectively.

X-Ray Crystallography—Crystals of wild-type EqADH complexed with NADH and (R)-N-1-methylhexylformamide were prepared as described by the general procedure used previously (19). Recrystallized horse liver enzyme from Boehringer Mannheim (about 0.5 ml of 9.4 mg/ml) was dialyzed at 4 ºC against 10 ml of 50 mM ammonium \( N\)-[tris(hydroxymethyl)methyl]-2-
aminoethanesulfonate, pH 6.7 (measured at 25 °C) with 1 mM NADH and 5 mM \((R)-N-1\)-methylhexylformamide, and the concentration of 2-methyl-2,4-pentanediol was raised over 24 days to 21%, when crystals formed. The concentration of diol was finally raised to 25% over 17 more days. X-ray data were collected at 100 K at the IMCA-CAT beam line at the Advanced Photon Source at Argonne National Laboratories with a wavelength of 1.000 Å, a crystal to detector distance of 140 nm, an exposure time of 1 s, and an oscillation angle of 0.3 Å, for a total of 600 images. The data were processed with DEN20 and SCALEPACK (20). The structure was solved by molecular replacement using AMORE (21) and the coordinates for the refined wild-type horse ADH-NAD\(^+\)-2,3,4,5,6-pentaflourobenzyl alcohol complex (PDB entry 1hld) as a model. The structure was refined by cycles of restrained refinement using REFMAC (22) and model building with the program O (23). The initial electron density maps were calculated without including the formamide, but after one cycle of refinement, the positions for the formamides in all four subunits were readily apparent. Initially, the dictionary used for NADH maintained planarity of the nicotinamide ring, but the electron density maps clearly showed that the ring was not planar. Therefore, during the final cycles of refinement, the dictionary for NADH was modified to remove the restraints on planarity and torsion angles, as would be appropriate for NADH.

**Biological Evaluation**—The effects of the inhibitors on ethanol metabolism in mice were studied by the following procedure. Fed, male Swiss-Webster mice (20–25 g, obtained from Harlan-Sprague, Indianapolis, Indiana, USA) were administered intraperitoneal injections of the inhibitor in a dose of 0 to about 1 mmol/kg of body weight. The solutions of inhibitor were 0.1 M or lower in physiological saline. Ethanol (4 M in physiological saline) was administered 20-30 min later, by intraperitoneal injection at 65 mmol/kg of body weight. Blood samples (10 µl) were
drawn from the tail at intervals timed from the ethanol injection. The blood alcohol concentration was determined by gas chromatography as previously described (10).

RESULTS

Evaluation of Inhibitors with Purified Enzymes—We determined the inhibition constants for the series of N-substituted formamides, sulfoxides, and pyrazoles by initial velocity studies with varied concentrations of the inhibitor and a substrate appropriate for each purified alcohol dehydrogenase. Formamides produce uncompetitive inhibition against varied concentrations of ethanol and competitive inhibition against varied concentrations of cyclohexanone (Fig. 1). Previous studies showed that the $K_i$ values are not dependent on the substrate used and that the inhibition constants determined for the forward or reverse reaction were about (within 2-fold) the same (10, 13-15). The simplest kinetic explanation for the inhibition results is the Ordered Bi Bi mechanism where the inhibitor binds only to the enzyme-NADH complex. For this mechanism, the $K_i$ values represent true dissociation constants since the concentrations of coenzyme were saturating, and dissociation of NADH is rate-limiting for turnover (10).

The inhibition constants for $EqADH$, $MmADH1$, and $HsADH1C*2$ are presented in Table I. The $K_i$ values for most of the formamides ranged from about 1 to 100 µM, indicating potent inhibition. These enzymes have a few differences of amino acid residues in the active sites and thus different specificities, which are reflected in $K_i$ values that can differ by 10-fold or more among the enzymes. It is significant, for instance, that the linear N-n-propyl- and N-n-butylformamides (1, 2) bind more tightly to $EqADH$ and $MmADH1$ than to $HsADH$, whereas the secondary formamides that are branched at the one position (7, 8, 9, 10, 11, 12, 13, 14 and 20) bind more tightly to $MmADH1$ and $HsADH$ than to $EqADH$. ($R$)-N-1-Methylhexylformamide (7), its heptyl analogue (10) and N-1,5-dimethylhexylformamide (11) are particularly potent
inhibitors of the human and mouse enzymes, with $K_i$ values less than 1 µM. The methylated cyclohexylformamides are also better inhibitors of $Mm$ADH1 and $Hs$ADH than of $Eq$ADH. It appears that $Mm$ADH1 and $Hs$ADH have more room in the active site than does $Eq$ADH.

We also determined inhibition constants for sulfoxides and pyrazoles with $Mm$ADH1. The sulfoxides bind to the E–NADH complex and are uncompetitive inhibitors against varied concentrations of alcohol, whereas the pyrazoles bind to the E–NAD$^+$ complex and are competitive inhibitors. The values for $Mm$ADH1 are similar to those observed for $Eq$ADH, $Hs$ADH1C*2, and rat ADH (10, 11, 15). The 3-substituted thiolane 1-oxides (alkyl derivatives of the 5-membered tetramethylene sulfoxide ring) are potent inhibitors of all of the enzymes, whereas the methyl alkyl sulfoxides are much less potent. The 4-substituted pyrazoles are also potent inhibitors.

X-ray Crystallography—The structure of $Eq$ADH complexed with NADH and (R)-N-1-methylhexylformamide was determined at 1.57 Å resolution. The X-ray data collection and refinement statistics are summarized in Table II. The asymmetric unit contained two molecules of enzyme; all four subunits have very similar structures, which are very similar to those of other ADH complexes, and are in the closed conformation (13, 19, 24). The location of the formamide is well-defined in the electron density maps (Fig. 2A). The results show how the enzyme binds the aldehyde analogue and accommodates the hexyl group.

The geometry of binding suggests that the formamide mimics the Michaelis complex with NADH and aldehyde (Fig. 2B). Significantly, the nicotinamide ring of the NADH-(R)-N-1-methylhexylformamide complex is in a boat conformation where the N1 and C4 atoms are not in the same plane as atoms C2, C3, C5, and C6. The puckering of the nicotinamide ring can be described by the angle $\alpha$N1 between the planes defined by atoms C2-N1-C6 and C2-C3-C6,
angle $\alpha$C4 between the planes defined by atoms C3-C4-C5 and C2-C3-C6, and the twist (distortion of the boat) calculated as the distance in angstroms between C5 and the plane defined by atoms C2-C3-C6 (25). For the formamide complex, $\alpha$N1 is $12 \pm 2^\circ$, $\alpha$C4 is $18 \pm 4^\circ$, and twist is $1.2 \pm 0.7$ Å, mean values for the four subunits.

*Biological Evaluation in Mice*—The effects of five selected inhibitors on the rate of metabolism of ethanol were studied with mice, and the progress curves were fitted to an overall mechanism that provides *in vivo* inhibition constants and rate constants for the time-dependent elimination of the inhibitor. (In this context, we use the term “elimination” to include the metabolism and excretion of the alcohol and inhibitors.) The results presented in Fig. 3 show that N-1-methylhexylformamide significantly decreases the rate of elimination of ethanol. In the absence of inhibitor, the kinetics are zero-order, which is due to the saturation of the alcohol metabolizing system (i.e., it is at maximum velocity) and is typical of animals (26). In the presence of inhibitor, the time required for complete elimination of alcohol is substantially longer, showing that inhibitors are active *in vivo*. However, the progress curves are not linear, probably due to elimination of the inhibitor and decreased efficacy over time. Thus, elimination rates and *in vivo* $K_i$ values are not easily calculated. Previous studies used estimates of the initial velocity of ethanol elimination in order to obtain $K_i$ values (10-12). Advances in computing now provide a more robust method of analysis.

Numerical integration was used to fit the full progress curves to a mechanism that describes the overall process (27, 28), and the apparent *in vivo* $K_i$ values and the rates of elimination of the inhibitors were determined. The mechanism used for the simulation is given in Fig. 4. The Ordered Bi Bi reaction of alcohol dehydrogenase is modified by including the binding of the inhibitor (I) to the enzyme-NADH complex to form E-NADH-I, which accounts for the
uncompetitive inhibition against alcohol. The elimination of inhibitor (I → J) by a first order process is expressed as an additional reaction. Previous studies that directly determined the concentration of inhibitor in blood of rats gave a first order rate constant for isobutyramide of 0.042 min⁻¹ (12) and for tetramethylene sulfoxide of 0.075 min⁻¹ (10). Although ADH is the major enzyme system involved in ethanol metabolism (~ 90%), other metabolic pathways such as a cytochrome P450 (CYP2E1) and excretion can account for elimination of ethanol when the ADH inhibitor is saturating. In rats, non-ADH pathways were estimated to contribute about 10% of the total rate and to have an apparent $K_m$ value for ethanol of 21 mM (12). Sophisticated isotopic methods also show that alcohol dehydrogenase accounts for the metabolism of at least 89% of the ethanol (29, 30). Thus, the reaction of enzyme F is added to the overall mechanism.

Many of the rate constants for the mechanism in Fig. 4 can be estimated from kinetic constants that have been determined for liver alcohol dehydrogenases (31, 32). Since ADH is typically saturated with substrates during ethanol metabolism, the turnover number ($V_{\text{max}}$) of ADH controls the rate of ethanol metabolism, and this is usually controlled by the rate-limiting release of NADH, $k_{-4}$, which is the only sensitive rate constant for the ADH reaction. The maximum rate of ethanol elimination is the product of enzyme concentration and $k_{+4}$. The concentration of ADH is calculated from the rate of ethanol elimination in the absence of inhibitor. (The concentration of ADH is calculated for the whole body mass and is consistent with measurements based on ADH activity in the liver and the fraction of body mass that is liver (33, 34)). Similarly, the concentration of enzyme F and its turnover number ($k_{+8}$) are set to account for 10% of the overall rate of ethanol elimination at the dose of ethanol used.

The data in Fig. 3 are described well by the mechanism in Fig. 4, and the estimates of the fitted parameters are good (Table III). All of the inhibitors are effective in vivo. The rates of
elimination of the inhibitors determined from these data are comparable to those determined from analysis of the remaining compounds in blood levels during the ethanol elimination (10, 12). However, the \textit{in vivo} $K_i$ values are typically about an order of magnitude larger than the ones determined \textit{in vitro} with isolated enzyme. Previous studies showed that inhibition by tetramethylenesulfoxide, isobutyramide, isovaleramide, and 4-methylpyrazole also was characterized by larger $K_i$ values \textit{in vivo} as compared to \textit{in vitro} (10, 12). The differences are not due to the different units being used (moles/liter \textit{in vitro}; moles/kg \textit{in vivo}) since the animal has about 70% body water (0.7 ml/g) as determined from the concentration of ethanol at zero time (moles/liter) as compared to the dose of ethanol (moles/kg). The differences do not appear to be due to an incorrect mechanism as previous studies showed that the types of inhibition by these compounds are essentially the same \textit{in vitro} and \textit{in vivo} (10, 12). The differences are not due to metabolism of the inhibitors as the analysis of progress curves accounts for the elimination, and initial velocity analysis gives similar results. A simple explanation is that the bioavailability of the inhibitors in the animals reduces the effective concentration of free inhibitor and thereby increases the apparent $K_i$ values. If the compounds were preferentially absorbed in extrahepatic tissues or membranes, the concentration of free inhibitor in the liver cytosol would be reduced.

Potent inhibition \textit{in vivo} requires a compromise in lipophilicity that is optimized for affinity for ADH and bioavailability. Increasing the size of the aliphatic side chain, from isopropyl to methylhexyl for instance, decreases the \textit{in vitro} $K_i$, but has little effect on the \textit{in vivo} $K_i$ (Table III). Similarly, 3-butylthiolane 1-oxide is a better inhibitor \textit{in vivo} than the more hydrophobic 3-phenyl and 3-hexyl derivatives or the less hydrophobic tetramethylene sulfoxide and 3-methylthiolane 1-oxide (11).
Toxicity of the formamides was not directly determined, but during the studies on the inhibition of ethanol metabolism, the mice survived for at least one day after the experiments with the highest doses (in mmoles/kg) being 0.36 for N-isopropylformamide, 0.32 for N-isopentylformamide, 0.30 for N-1-methylhexylformamide, 0.40 for N-cyclohexylformamide, and 0.36 for 3-butythiolane 1-oxide. However, at doses of 0.72 mmoles/kg of N-isopentylformamide, 0.80 of N-cyclohexylformamide, 0.60 of N-1-methylheptylformamide, and 0.72 of 3-butythiolane 1-oxide, toxicity was suspected because the rates of ethanol metabolism were depressed more than expected from the studies with lower doses of inhibitor. Furthermore, a dose of 1.8 mmole/kg of N-1-methylhexylformamide was lethal within 30 min.

The results show that the short chain N-isopropylformamide is about as potent in vivo as the longer chain compounds. The relatively slow rate of elimination of N-isopropylformamide is a potential advantage as compared to the other inhibitors. N-Cyclohexylformamide and 3-butythiolane 1-oxide are also potent inhibitors, but their rates of elimination are faster.

DISCUSSION

Structure of the Michaelis Complex Analogue—The structure of the enzyme-NADH-(R)-N-1-methylhexylformamide complex appears to resemble the ground state for the NADH-aldehyde complex. The structural features are relevant for understanding the catalytic mechanism. The formamide oxygen is ligated to the catalytic zinc at a distance of 2.2 Å (Fig. 2A). The oxygen forms a hydrogen bond to the hydroxyl group of Ser-48, which is connected to the 2'-hydroxyl group of the nicotinamide ribose and eventually to His-51, which is on the surface of the enzyme (19). This system appears to relay a proton between substrate and His-51, which acts as an acid/base catalyst (19, 35-37). The inhibitor binds with a cis conformation, which produces a potential cation-π interaction between the amide N-H and the benzene ring of Phe-93 of the
enzyme. The formyl group of the formamide is oriented as would be expected for direct transfer of hydrogen from the nicotinamide ring of the coenzyme to the \textit{re} face of the carbonyl carbon (Fig. 2B). The distance between the carbonyl carbon of the formamide and C4N of NADH is 3.6 Å, which is reasonable for the ground state for hydride transfer.

The puckering of the nicotinamide ring of NADH has not yet been observed in any other structure of a complex of a dehydrogenase with NADH and a substrate or substrate analogue. A twisted boat conformation has been observed for a complex of \textit{Eq}ADH with NADH and a bound water, but the relevance of the complex for the catalytic mechanism is not clear (25). The nicotinamide ring is also puckered in the complex with NAD$^+$ and pyrazole, which form a partial covalent bond between C4 of the nicotinamide ring and a N from pyrazole and may mimic the transition state of the reaction (38). Molecular dynamics simulations on the native ADH-NADH-benzaldehyde complex show fluctuations of the protein cause the nicotinamide ring of NADH to bend, with angles for the C4 deviation being as large as 20° (39). Calculations show that puckering of the dihydronicotinamide ring, to form a quasi-boat conformation, decreases the transition state energy for hydrogen transfer of the pseudoaxial hydrogen at C4 (40, 41). The structure of the complex with NADH and the formamide suggests that the ground state can have a puckered nicotinamide ring.

\textit{Structure-Function Relationships}—These studies provide data for the design of potential therapeutic agents and for correlating structure and function of the liver alcohol dehydrogenases. The inhibition constants determined in this study are thermodynamic dissociation constants that reflect the energetics of multiple binding interactions. Thus, the binding of the inhibitors is a measure of substrate specificity.
In order to explain the results in Table I, we docked models of the various inhibitors into the active sites of the enzyme-coenzyme complexes. We used the structures of \textit{EqADH} with NADH and (\textit{R})-N-1-methylhexylformamide or \textit{N}-cyclohexylformamide (1LDY.PDB, ref. 13). Structures of the mouse and human enzymes complexed with these formamides are not available, but there is a structure of the human liver alcohol dehydrogenase with coenzyme (1HTO.PDB, ref. 42), and a model of the mouse enzyme was constructed based on the structure of \textit{EqADH} (1HLD.PDB). These three enzymes share 84-89\% sequence identity, and the amino acid residues lining the substrate binding sites are identical, except at positions 110 and 141. As compared to Phe-110 and Leu-141 in the horse enzyme, the mouse enzyme has Ile-141, and the human enzyme has Tyr-110 and Val-141 (43). The substitutions at residue-141 should allow the substrate binding sites in the mouse and human enzymes to accommodate somewhat larger ligands. There are also other differences in the amino acid residues that make contact with the residues in the active site, and these may affect specificity through indirect effects on conformation and enzyme flexibility.

The amino acid residues in the substrate binding site are all aliphatic or aromatic (Fig. 2A). Only the catalytic zinc and Ser-48, which interact with the O of the substrate are polar. Thus, we expect that tight binding is associated with good van der Waals contacts or hydrophobic interactions, a lack of voids that would accommodate water molecules, and the absence of steric conflicts. We expect that an inhibitor that fills the space should bind optimally. Of course, the binding modes can vary due to flexible amino acid side chains that adapt to the ligand structure and optimize interactions (24).

The modeling shows that all of the formamides can fit, without bad steric contacts, into the substrate-binding sites, sometimes with small changes in positions of the amino acid side chains.
However, it does not appear that binding affinity simply reflects hydrophobic interactions, as there are only modest changes in affinity as the size of the inhibitor (number of carbons) is increased (Table I). For instance, increasing the chain length from $N$-isopropyl (4) to ($R$)-$N$-1-methyhexyl (7) slightly decreased affinity for the horse enzyme, whereas affinities for the mouse and human enzymes were increased by about an order of magnitude. In general, the mouse and human enzymes have higher affinities for the larger molecules, which might be attributed to the substitutions at residue 141 (42). Consistent with this explanation is that the $N$-1-ethylhexyl and $N$-1-ethylheptyl formamides (13, 14) bind less well than the $N$-1-methyl derivatives (9, 10) to the horse enzyme whereas they bind more tightly to the mouse and human enzymes. However, our modeling suggests that the $N$-1-ethyl derivatives can be accommodated in the horse enzyme in a position similar to the one observed for ($R$)-$N$-1-methyhexylformamide. Previous studies also suggested that the human enzyme could accommodate larger $N,N$-disubstituted, bicyclic formamides (14), and we conclude that the human enzyme has more flexibility to adapt to larger ligands than does the horse enzyme. In contrast, the $N$-n-propyl (1) and butyl (2) formamides bind more weakly to the human enzyme than to the horse enzyme, and this may be explained by the increased volume in the human enzyme that allows a water molecule to infiltrate and disrupt hydrophobic interactions. The factors that control binding affinity are subtle and require further studies to provide quantitative explanations.

In principle, enzymes are stereoselective for binding of inhibitors, and the horse enzyme binds the $1S$ isomers of 3-butylthiolane 1-oxide at least 10-fold more tightly than the $1R$ isomers (24). In contrast, the binding of the $R$ and $S$ isomers of $N$-1-methyhexylformamide shows a moderate preference for the $R$ isomer. Both isomers can be modeled into the binding sites without producing bad steric contacts. Similarly, the isomers of the other chiral inhibitors (5, 6,
10, 11, 13, 14, 16, and 17, studied as racemic mixtures) could fit into the active sites. Adding methyl groups to the cyclohexyl rings (16, 17) did not improve binding. Further work is required to define the stereochemical preferences of the active site.

As previously determined for the horse and human enzymes, the methyl alkyl sulfoxides are less potent inhibitors than the cyclic thiolane 1-oxides, for which structures of the complexes with alcohol dehydrogenase have been determined by X-ray crystallography (24). Structures for the horse and human enzymes with pyrazole and 4-iodopyrazoles have been determined (38, 44, 45, 46). The especially high affinity of the pyrazoles is due to the formation of a partial covalent bond, mimicking the transition state, with the bound NAD$^+$ (38).

**Biological Relevance**—Liver alcohol dehydrogenase is the predominant rate-determining factor in the metabolism of ethanol and therefore a good target for inhibitory drugs (2, 10, 47). Since the Class I enzymes can detoxify various biogenic and dietary alcohols and aldehydes, we directed our studies to these enzymes. Recent gene knockout studies in mice provide compelling information that the ADH1 is the major enzyme involved in ethanol metabolism (48). Mice lacking a functional ADH1 gene have a significantly decreased rate of elimination of an intoxicating dose of ethanol. Isopropylformamide, cyclohexylformamide and 3-butylthiolane 1-oxide are good inhibitors of mouse ADH1 *in vitro* and are effective *in vivo*. These results support the conclusion that ADH1 is a major factor in ethanol metabolism and the proposal that the inhibitors are relatively specific.

These compounds are relatively non-toxic. The LD$_{50}$ for *N*-cyclohexylformamide is 2.5 mmole/kg (14), and that for 3-butylthiolane 1-oxide is 1.4 mmole/kg (11). Since the effectiveness of inhibition also depends upon the rates of metabolism of the inhibitors, the results on the apparent rate of elimination can be used to devise protocols for repetitive dosing. The
formamides and 3-substituted thiolane 1-oxides are almost as potent as the competitive inhibitor 4-methylpyrazole, which is being used to treat methanol poisoning, but they are uncompetitive inhibitors against varied concentrations of ethanol and thus are effective even at saturating concentrations of alcohol. These compounds should be useful for studies on the metabolism of various alcohols and for treatment of poisoning due to methanol or ethylene glycol.

Acknowledgements—We thank S. Ramaswamy for collecting the x-ray data and for assistance with the refinement of the structure and Daniel J. Ferraro for calculating the geometry of the nicotinamide rings. We thank Kristine B. Berst for assistance with the kinetic determinations. We thank Dr. Jan-Olov Höög for the expression vector for HsADH1C*2 and Dr. Gregg Duester for the expression vector for MmADH1. Synchrotron data were collected at beamline 17-ID in the facilities of the Industrial Macromolecular Crystallography Association Collaborative Access Team (IMCA-CAT) at the Advanced Photon Source. These facilities are supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with Illinois Institute of Technology (IIT), executed through IIT's Center for Synchrotron Radiation Research and Instrumentation. Use of the Advanced Photon Source at Argonne National Laboratory was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under contract No. W-31-109-Eng-38.
REFERENCES


Textual Footnotes:

1 The abbreviations used are: ADH, alcohol dehydrogenase; HsADH1C*2, human (*Homo sapiens*) ADHγ2γ2 (49); EqADH, horse (*Equus caballus*) ADH, EE (ethanol active isoenzyme); MmADH1, mouse (*Mus musculus*) enzyme.
FIGURE LEGENDS

FIG. 1. Inhibition of mouse ADH1 and human ADH1C*2 by N-1,5-dimethylhexylformamide. The inhibition was studied at 37 °C in 83 mM potassium phosphate, 40 mM KCl and 0.25 mM EDTA buffer, pH 7.3. A, Inhibition of mouse ADH1 with ethanol as varied substrate. The concentrations of formamide were 0 (●), 0.25 (■), 0.5 (▲), and 1 (◆) µM, and the concentration of NAD⁺ was 2 mM. The data were fitted to the equation for uncompetitive inhibition, \( \nu = \frac{VA}{K_m + A(1 + I/K_i)} \), where \( V \) is the maximum velocity and \( A \) is the varied substrate. B, Inhibition of human ADH1C*2 with cyclohexanone as varied substrate. The concentrations of formamide were 0 (●), 1.25 (◆), 2.5 (▲), and 5 (■) µM, and the concentration of NADH was 0.2 mM. The data were fitted to the equation for competitive inhibition, \( \nu = \frac{VA}{K_m(1 + I/K_i) + A} \). The standard errors of the fits were less than 10%.

FIG. 2. Active site of horse liver alcohol dehydrogenase complexed with NADH and (R)-N-1-methylhexylformamide. A, An electron density map (2\(F_o\) - \(F_c\)) was calculated at one standard deviation above the mean including the atoms of the inhibitor with the refined structure. B, Top view of the active site with dashed lines indicating the hydrogen bonds of the proton relay system. This view also shows the puckering of the dihydronicotinamide ring.

FIG. 3. Inhibition of ethanol metabolism in mice by N-1-methylhexylformamide. Mice were given varied doses of formamide and 10 min later a dose of 65 mmole/kg ethanol. The blood alcohol concentration was determined at various times. A total of five animals were used, with different concentrations of the inhibitor: 0 (●), 100 (■), 300 (◆), 600 (▲) µmoles/kg. All the data were fitted together using the mechanism given in Fig. 4 and all rate constants fixed except for \( k_{-5} \) and \( k_{+6} \). The concentration of E (ADH) was set at 0.15 µM, enzyme F at 0.1 µM, and the concentration of NAD⁺ was 2 mM. The data were fitted to the equation for uncompetitive inhibition, \( \nu = \frac{VA}{K_m + A(1 + I/K_i)} \), where \( V \) is the maximum velocity and \( A \) is the varied substrate.
NAD$^+$ at a fixed steady-state level of 0.5 mM, NADH at a fixed steady-state level of 2 µM, and the initial concentration of ethanol at 95 mM. The concentration of acetaldehyde is negligible due to rapid oxidation by aldehyde dehydrogenases (34). Fixed rate constants were as follows: $k_{+1}$, 8.4 x 10$^7$ M$^{-1}$min$^{-1}$; $k_{-1}$, 9000 min$^{-1}$; $k_{+2}$, 1.4 x 10$^7$ M$^{-1}$min$^{-1}$; $k_{-2}$, 3.1 x 10$^4$ min$^{-1}$; $k_{+3}$, 8200 min$^{-1}$; $k_{-3}$, 3.1 x 10$^4$ min$^{-1}$; $k_{+4}$, 3600 min$^{-1}$; $k_{-4}$, 1.3 x 10$^9$ M$^{-1}$min$^{-1}$; $k_{+5}$, 1 x 10$^6$ M$^{-1}$min$^{-1}$; $k_{-5}$, 6 x 10$^7$ M$^{-1}$min$^{-1}$; $k_{-7}$, 1.3 x 10$^6$ min$^{-1}$; and $k_{+8}$, 450 min$^{-1}$. See Ref. 28 for a detailed file for the coding for KINSIM.

**FIG. 4.** Mechanism for describing the inhibition of alcohol metabolism by an uncompetitive inhibitor, which is slowly eliminated.
Table I

Inhibition of purified liver alcohol dehydrogenases by formamides, sulfoxides and pyrazoles

The inhibition constants were determined in 83 mM potassium phosphate, 40 mM KCl and 0.25 mM EDTA buffer, pH 7.3, at 37 °C by measuring the change in absorbance of NADH at 340 nm. Buffers for the determinations cited from the literature were 46 mM sodium phosphate, pH 7.0 and 25 °C. Substrates for the enzymes were chosen for optimal activity. The coenzyme concentrations were fixed at high, saturating levels (2.0 mM NAD⁺ or 0.2 mM NADH) while the concentrations of substrates and inhibitors were varied over at least a 3-fold range. Some 20–25 data points were collected for each inhibitor. The data were fitted to the equations for competitive or uncompetitive inhibition with the appropriate computer programs (50). Standard errors are usually less than 20% of the values.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (µM)</th>
<th>$EqADH^a$</th>
<th>$MmADH1^a$</th>
<th>$HsADH1C^b$</th>
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<tr>
<td>1 N-n-propylformamide</td>
<td>10^c</td>
<td>6.4</td>
<td>120^c</td>
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</tr>
<tr>
<td>2 N-n-butylformamide</td>
<td>16^c</td>
<td>12</td>
<td>100^c</td>
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</tr>
<tr>
<td>3 N-isopentylformamide</td>
<td>80</td>
<td>7.6</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>4 N-isopropylformamide</td>
<td>2.6^c</td>
<td>6.1</td>
<td>15^c</td>
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<tr>
<td>5 N-1,2-dimethylpropylformamide</td>
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<td>15</td>
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<tr>
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<td>0.41^c</td>
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<td>No.</td>
<td>Compound</td>
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<td>Kᵢ (µM)</td>
<td>Kᵢ/(IC₅₀)</td>
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<td>-----</td>
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<td>3-butylthiolane 1-oxide</td>
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<td>0.30&lt;f</td>
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<td>0.013&lt;h</td>
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<td>0.21&lt;i</td>
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<td>0.02&lt;h</td>
<td>0.12</td>
<td>0.12&lt;i</td>
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</tbody>
</table>

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*a* Inhibition constants were determined with ethanol (0.2–2 mM) as the varied substrate, and the data were fitted to the equation for uncompetitive inhibition.

*b* The varied substrate was cyclohexanone (1.5–12 mM), and the data were fitted to the equation for competitive inhibition.

*c, d, e, f, g, h* Values were obtained from refs. 14, 13, 10, 15, 11, and 51, respectively.

*i* Values from ref. 52 for the activity of a mixture of isoenzymes.
Table II

X-ray data and refinement statistics for horse liver alcohol dehydrogenase complexed with NADH and (R)-N-1-methylhexylformamide

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<td>Space group</td>
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<tr>
<td>Cell dimensions, Å</td>
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<tr>
<td>Dimeric molecules per unit cell</td>
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</tr>
<tr>
<td>Resolution range, Å</td>
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<tr>
<td>Measured reflections: unique, total</td>
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<td>Completeness, % (outer shell)</td>
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</tr>
<tr>
<td>(R_{\text{sym}}), % (outer shell)(^a)</td>
<td>4.7 (20)</td>
</tr>
<tr>
<td>Mean (&lt;I&gt;/\sigma(I)) (outer shell)</td>
<td>32.9 (5.2)</td>
</tr>
<tr>
<td>(R_{\text{value}}, R_{\text{free}}, \text{test %})(^b)</td>
<td>0.153, 0.200, 0.6</td>
</tr>
<tr>
<td>rmsd for bond distances, Å(^c)</td>
<td>0.014</td>
</tr>
<tr>
<td>rmsd for bond angles, deg(^c)</td>
<td>1.47</td>
</tr>
<tr>
<td>Number of water molecules</td>
<td>1241</td>
</tr>
</tbody>
</table>

\(^a\) \(R_{\text{sym}} = (\Sigma |I - <I>|)/\Sigma <I>\), where \(I\) is the integrated intensity of a given reflection.

\(^b\) \(R_{\text{value}} = (\Sigma |F_o - kF_c|)/\Sigma|F_o|\), where \(k\) is a scale factor. \(R_{\text{free}}\) (53) was calculated with the indicated percentage of reflections not used in the refinement.

\(^c\) Deviations from ideal geometry.
Table III

*Inhibition of ethanol metabolism in mice*

The parameters for inhibition *in vivo* were obtained by fitting the progress curves for ethanol elimination with varied concentrations of inhibitors. See Fig. 3 for representative results.

Correlation coefficients were typically 0.99 and standard errors for the parameters *in vivo* were less than 20%.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$in;vitro;K_i$ (µM)</th>
<th>$in;vivo;K_i$ (µmoles/kg body weight)</th>
<th>Rate Constant for Elimination of Inhibitor (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N$-isopropylformamide</td>
<td>6.1</td>
<td>110</td>
<td>0.00021</td>
</tr>
<tr>
<td>$N$-isopentylformamide</td>
<td>7.6</td>
<td>130</td>
<td>0.0094</td>
</tr>
<tr>
<td>$N$-1-methylhexylformamide</td>
<td>1.6</td>
<td>91</td>
<td>0.0080</td>
</tr>
<tr>
<td>$N$-cyclohexylformamide</td>
<td>2.5</td>
<td>32</td>
<td>0.012</td>
</tr>
<tr>
<td>3-butylthiolane 1-oxide</td>
<td>0.40</td>
<td>9.7</td>
<td>0.022</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Alcohol Dehydrogenase Pathway

\[
\begin{align*}
E-NAD^+ & \xleftarrow{k+2} \text{alcohol} & \xrightarrow{k-2} E-NAD^+-alc \\
E-NADH & \xleftarrow{k-3} E-NADH-ald & \xrightarrow{k+3} \text{aldehyde} \\
E-NADH-I \quad \quad k-5 & \xrightarrow{k+5} I \\
& k+6 & \rightarrow J
\end{align*}
\]

Elimination of Inhibitor

Non-ADH Pathway

\[
\begin{align*}
F & \xleftarrow{k+7} FB & \xrightarrow{k-7} F + \text{Product} \\
FB & \xrightarrow{k+8} F + \text{Product}
\end{align*}
\]

Figure 4
Formamides mimic aldehydes and inhibit liver alcohol dehydrogenases and ethanol metabolism
Thulasiram H. Venkataramaiah and Bryce V. Plapp

J. Biol. Chem. published online July 10, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305419200

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