Binding of Formamides to Liver Alcohol Dehydrogenase[†,‡]

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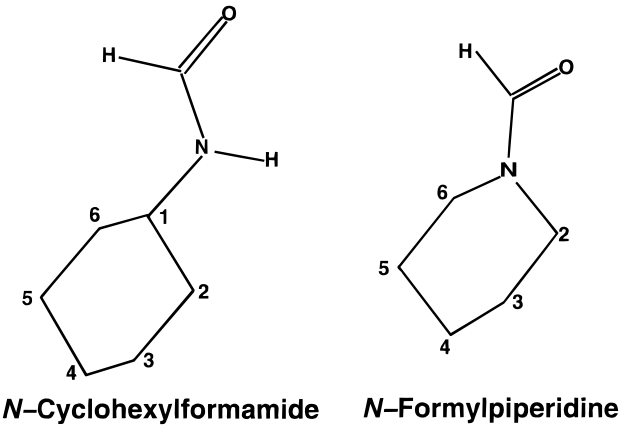
ABSTRACT: Amides are analogs of aldehydes and potent inhibitors of liver alcohol dehydrogenases. They can be used for structural studies and for inhibiting the metabolism of alcohols that form toxic products. We studied *N*-alkyl amides that bind to the enzyme−NADH complex and act as uncompetitive inhibitors against varied concentrations of ethanol (millimolar K_{ii} values, at pH 8 and 25 °C): *N*-propylacetamide (16), δ-valerolactam (1.6), *N*-formylpiperidine (0.14), *N*-isobutylformamide (0.028), *N*-(cyclohexylmethyl)-formamide (0.011), and *N*-cyclohexylformamide (0.0087). The lower affinity of δ-valerolactam and *N*-propylacetamide can be explained by steric hindrance with Phe93 of the enzyme. Replacing Phe93 with Ala in the S48T/F93A mutated enzyme, which resembles the natural α-isoenzyme of primates, improved binding of δ-valerolactam by 210-fold. The structures of horse liver enzyme complexed with NADH and *N*-cyclohexylformamide or *N*-formylpiperidine were determined by X-ray crystallography at 2.5 Å resolution. In both complexes, the carbonyl oxygens of the inhibitors bind to the catalytic zinc and form a hydrogen bond to the hydroxyl group of Ser48 of the enzyme. The six-membered rings bind in overlapping, but rotated, positions that optimize hydrophobic interactions. The binding modes of the unreactive formamides appear to resemble the Michaelis complexes of the analogous substrates, with the *re* face of the carbonyl carbon suitably positioned to accept a hydrogen from NADH.

X-ray crystallography of enzymes complexed with substrates or inhibitors that closely resemble substrates can provide important information about binding modes and reaction mechanisms. High-resolution structures of horse liver alcohol dehydrogenase complexed with NAD^+ and substituted benzyl alcohols or NADH and dimethyl sulfoxide have been determined (Ramaswamy et al., 1994; Al-Karadaghi et al., 1994), but structures with aldehydes, or close analogs, are needed. A structure at 2.9 Å resolution of the enzyme complexed with 1,4,5,6-tetrahydronicotinamide adenine dinucleotide (H_2NADH) and *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde was determined, but the crystals were prepared at pH 9.5 where the enzyme only slowly reduces the aldehyde with NADH (Cedergren-Zeppezauer et al., 1982). At pH 7, the equilibrium position for reactants bound to the active site favors NAD^+ and alcohol (Shearer et al., 1993). Thus, a suitable aldehyde analog would be useful for structural studies. Formamides might be appropriate as they are isosteric, unreactive analogs of aldehydes. Oxamate, for example, was used as an analog of pyruvate for X-ray crystallography of lactate dehydrogenase (Wigley et al., 1992).

Amides and formamides inhibit liver alcohol dehydrogenase and bind preferentially to the enzyme−NADH complex (Winer & Theorell, 1960; Sigman, 1967; Sarma & Woronick, 1972; Porter et al., 1976; Chadha et al., 1983; Delmas et al., 1983; Freudenreich et al., 1984). The amides inhibit alcohol metabolism and might be useful therapeutic agents, for example to prevent the oxidation of methanol or ethylene glycol to their toxic acids (Porter et al., 1976; Delmas et al., 1983; Plapp et al., 1984). As uncompetitive inhibitors against varied alcohol concentrations, the amides remain effective even with saturating concentrations of alcohols. We examined structure−function relationships for some amides and determined three-dimensional structures for horse liver alcohol dehydrogenase complexed with NADH and two formamides, whose structures are shown in Chart 1. The results provide a model for binding of aldehydes and illustrate the potential for rational design of inhibitors for isoenzymes of alcohol dehydrogenase.

Chart 1



***N*–Cyclohexylformamide** ***N*–Formylpiperidine**

EXPERIMENTAL PROCEDURES

Materials. Liver alcohol dehydrogenase (EC 1.1.1.1) was obtained from Boehringer Mannheim (Germany) and was

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[‡] The X-ray coordinates and structure factors for the complexes with *N*-cyclohexylformamide and *N*-formylpiperidine have been deposited in the Brookhaven Protein Data Bank with entry names 1LDY, r1ldysf, 1LDE, and r1ldesf, respectively.

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recrystallized with ethanol. The S48T/F93A mutated alcohol dehydrogenase was prepared as described by Kim (1994).

Amides and reagents were supplied by Aldrich, except *N*-isobutylformamide and *N*-(cyclohexylmethyl)formamide, which were prepared by refluxing the corresponding amine with formic acid, extracting with chloroform, and distilling under reduced pressure. Compounds were characterized using a Varian 360 MHz NMR spectrometer. *N*-Isobutylformamide: yield 55%; bp 118−120 °C (10 mm); ^{1}H-NMR ($CDCl_3$) δ 0.89 (d, CH_3), 1.74 and 1.8 (m, CH), 3.03 and 3.14 (t, CH_2), 5.79 and 5.95 (broad, NH), 8.02 (d), 8.2 (s, CHO). Multiple resonances are due to the *cis*−*trans* isomerism with a ratio of about 3:1. *N*-(Cyclohexylmethyl)formamide: yield 48%; bp 112−114 °C (3 mm); ^{1}H-NMR (DMSO-d_6) δ 0.87 (m, ring-CH_2), 1.17 (m, ring-CH_2), 1.37 (m, CH), 1.65 (m, ring-CH_2), 2.95 (N-CH_2), 3.3 (s, NH), 8.00 (s, CHO). *N*-Propylacetamide was obtained by reaction of 1-propylamine and acetyl chloride in 1,4-dioxane and distillation: yield 51%; bp 119−120 °C (3 mm); ^{1}H-NMR ($CDCl_3$) δ 0.93 (t, propyl-CH_3), 1.55 (m, CH_2), 2.05 (s, acetyl-CH_3), 3.22 (q, N-CH_2), 6.6 (broad, NH).

Kinetics. Kinetic constants were determined in 33 mM sodium phosphate and 0.25 mM EDTA buffer at pH 8 and 25 °C by measuring the change of absorbance at 340 nm due to NADH. Coenzyme concentrations were saturating at 2 mM NAD^+ or 0.1 mM NADH with 0.12−0.75 mM ethanol or 0.2−1 mM acetaldehyde. Data were analyzed with the appropriate FORTRAN programs (Cleland, 1979).

Crystallography. Alcohol dehydrogenase (10 mg/mL) was dialyzed in 1 mL bags against 10 mL of 50 mM ammonium *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonate buffer (pH 7.0) at 5 °C (pH 6.7 at 25 °C), 0.66 mM NADH, and 10 mM *N*-cyclohexylformamide or 14 mM *N*-formylpiperidine. Crystals formed after slow, cumulative addition of about 10% 2-methyl-2,4-pentanediol to the outer dialyzate and were brought to a final concentration of 25% diol over 8 weeks. Crystals were flash-frozen at 100 K in a stream of N_2. X-ray data for the complex with *N*-formylpiperidine were collected to a resolution of 2.5 Å on a Rigaku R-axis II imaging plate system with a rotating anode source. The data for the complex with *N*-cyclohexylformamide were collected on the X31 beam line at the EMBL DESY, in Hamburg, on a MAR image plate. The crystals belong to the monoclinic space group $P2_1$ with unit cell dimensions of $a = 49.93$ Å, $b = 180.22$ Å, $c = 86.79$ Å, and $\beta = 106.0°$ and two dimeric molecules of 80 kDa (four subunits) per asymmetric unit. The data were processed with the program DENZO and scaled with SCALEPACK (Otwinowski, 1993). The data for the complex with *N*-cyclohexylformamide were anisotropically scaled (Sheriff & Hendrickson, 1987) using the program ANISOB (CCP4 Suite, 1994). Amplitudes were computed from intensities using the program TRUNCATE (CCP4 Suite, 1994).

The initial starting model was the refined, isomorphous structure of the enzyme complexed with NADH and (1*S*,3*R*)-3-butylthiolane 1-oxide that was solved at 2.1 Å resolution (Brookhaven Protein Data Bank entry 1BTO; Cho et al., 1997). Rigid body refinement followed by several cycles of simulated annealing and positional and temperature factor refinement with XPLOR (version 3.1; Brünger et al., 1987) using strict noncrystallographic symmetry, but without including the formamides, produced structures with *R* values of about 25%. No constraints were applied to active site residues, except for the usual geometric restraints. (For the complex with *N*-cyclohexylformamide, anisotropic scaling was applied after the *R* value had decreased to a limiting value of 29% and thereafter refinement successfully continued.) At this stage, electron density maps, which were averaged over all four subunits with DM (Cowtan, 1994), clearly indicated the positions for the inhibitors (as illustrated in Figure 2). The program O was used for model building (Jones et al., 1991). Structures of the inhibitors were created and refined with energy minimization using SYBYL (TRIPOS Associates) and modeled into the active site. Then the structures were refined again (simulated annealing, positional and temperature factor refinement) using XPLOR with strict NCS restraints. Topology and parameter files were generated with approximate Engh and Huber parameters using the program XPLO2D written by G. Kleywegt (Uppsala, Sweden). Further refinement was accomplished by using the programs REFMAC with NCS restraints (Murshadov et al., 1996), which calculates the maximum likelihood of phases, and ARP (Lamzin & Wilson, 1993), which automatically adds or deletes waters. The latter step removed waters that were present in the starting model at 2.1 Å, but not visible at 2.5 Å. Electron density maps ($2|F_o| - |F_c|$) were used to adjust atomic positions, and finally, $|F_o| - |F_c|$ maps were found to be featureless, except for some surface residues that have alternative positions. Analysis with PROCHECK (Laskowski et al., 1993) and WHATIF using the Biotech Validation Server at EMBL-Heidelberg showed that good stereochemistry was obtained (*g* values of −0.30 for the complex with *N*-cyclohexylformamide and −0.58 for the complex with *N*-formylpiperidine). All amino acid residues were in allowed regions on the Ramachandran plot except for Cys174, which has been previously reported (Ramaswamy et al., 1994). Table 1 summarizes the crystallography. Illustrations of the structures were prepared with BOBSCRIPT, a modified version of MOLSCRIPT (Kraulis, 1991).

Table 1: Data Collection and Refinement Statistics for Complexes of Liver Alcohol Dehydrogenase with NADH and Formamides

	N-cyclohexylformamide	*N*-formylpiperidine
space group	$P2_1$	$P2_1$
total reflections measured	122 575	94 632
R_{merge} (%)[a]	4.2	3.5
unique reflections used	46 126	44 760
% completeness	92.9	88.8
resolution (Å)	12.0−2.5	20.0−2.5
$\langle I\rangle/\sigma\langle I\rangle$ at 2.54−2.50 Å	12.8	8.66
R_{value}, R_{free} (%)[b]	20.7	20.5, 25.6
bond distances[c]	0.02	0.02
bond angles[c]	2.6	2.0
water molecules[d]	1012	556

[a] $R_{merge} = (\sum|I - \langle I\rangle|)/(\sum\langle I\rangle)$. [b] $R_{value} = (\sum|F_o - kF_c|)/(\sum|F_o|)$, where *k* is a scale factor. The R_{free} value was calculated with 3% of the reflections not used in the refinement. For the complex with *N*-cyclohexylformamide, R_{free} was not calculated, due to the anisotropic scaling. [c] Root-mean-square deviations (rmsds) from the ideal geometry of the final model. [d] The complexes were refined with a starting model solved at 2.1 Å with 169 waters per subunit.

RESULTS AND DISCUSSION

Kinetics. The amides were uncompetitive inhibitors against ethanol and competitive against acetaldehyde (Table 2). This pattern is expected for the ordered bi-bi mechanism,

Table 2: Inhibition Constants for Horse Liver Alcohol Dehydrogenases[a]

	wild type		S48T/F93A
	ethanol[b]	acetaldehyde[c]	ethanol[b]
inhibitor	K_{ii} (μM)	K_{is} (μM)	K_{ii} (μM)
N-cyclohexylformamide	8.7 ± 0.6	7.1 ± 0.4	9.3 ± 0.8
N-(cyclohexylmethyl)formamide	11.5 ± 0.6	29 ± 3	103 ± 8
N-isobutylformamide	28 ± 2	14 ± 1	38 ± 11
N-formylpiperidine	139 ± 7	61 ± 3	8.7 ± 0.5
δ-valerolactam	1650 ± 150	8300 ± 780	7.6 ± 0.7
N-propylacetamide	16 900 ± 2400	57 000 ± 10 000	1200 ± 140

[a] Inhibition constants were determined at pH 8 and 25 °C with concentrations of inhibitor varied over at least a 3-fold range. The concentrations of coenzymes were saturating. [b] Inhibitors were uncompetitive (giving intercept inhibition constants, K_{ii}) against concentrations of ethanol varied from 0.12 to 0.75 mM with NAD^+ fixed at 2 mM. [c] Inhibitors were competitive (slope inhibition constants, K_{is}) against concentrations of acetaldehyde varied from 0.2 to 1 mM with NADH fixed at 0.1 mM.

with coenzymes binding before the other substrates and the inhibitor binding to the enzyme−NADH complex.

$$E \rightleftharpoons E{\cdot}NAD^+ \rightleftharpoons E{\cdot}NAD^+{\cdot}\,\text{alcohol} \rightleftharpoons E{\cdot}NADH{\cdot}\text{aldehyde} \rightleftharpoons E{\cdot}NADH \rightleftharpoons E$$

$$E{\cdot}NADH{\cdot}\,\text{amide}$$

The amides could also bind to free enzyme or the enzyme−NAD^+ complex, as found for other amides (Sigman, 1967; Sigman & Winer, 1970; Chadha et al., 1983). However, the competitive inhibition against acetaldehyde suggests that the binding is at least 10-fold weaker to E−NAD^+ than to E−NADH. It appears that the amides are good analogs of the aldehyde substrates. The formamides might be inhibitors of aldehyde dehydrogenases, also. The K_{ii} value for an amide was approximately the same as the K_{is} value (Table 2), as expected when saturating levels of coenzyme are used and the rate constants for coenzyme dissociation are rate-limiting steps in the mechanism.

Structure−Function Relationships. The K_i values for the *N*-monosubstituted formamides were in the range of 10−30 μM and similar to the values obtained for linear carboxamides and *N*-substituted formamides with the same number of carbon atoms (Winer & Theorell, 1960; Porter et al., 1976). Since the substrate binding pocket of horse liver alcohol dehydrogenase is a hydrophobic barrel (Eklund et al., 1981; Ramaswamy et al., 1994; Al-Karadaghi et al., 1994), the affinity for ligands generally increases with an increasing number of methylene units. The K_i values increased with *N*-disubstitution or substitution of the carbonyl carbon, which leads to crowding near the oxygen. *N*-Substituted formamides bind slightly better than do carboxamides of the same size (Porter et al., 1976). *N*-Methylformamide has a K_i value of 1.4 mM (Fan & Plapp, 1995), whereas acetamide has a K_i of 5 mM (Winer & Theorell, 1960) and dimethylformamide a K_i of 5.7 mM (Sharkawi, 1979). *N*-Methyl substitution of the amide nitrogen of benzamide also decreases the affinity (Sarma & Woronick, 1972). The higher inhibition constant of *N*-propylacetamide compared to that of δ-valerolactam is probably due to the entropic cost of binding the flexible, "open-chain analog".

In order to explain the relatively high inhibition constants for δ-valerolactam and *N*-propylacetamide, we docked them into the active site of the enzyme−coenzyme complex, using the structure derived from the ternary complex with NAD^+ and pentafluorobenzyl alcohol solved at 2.1 Å as a template (Ramaswamy et al., 1994). No structure of alcohol dehydrogenase complexed with amides (RCONHR′) is available, but the complex with pentafluorobenzyl alcohol is representative of several structures. The carbonyl oxygen (of the amide) was positioned as a ligand to the catalytic zinc and hydrogen bonded to the hydroxyl group of Ser48, as found in several ternary complexes. The NH of the amides was also positioned so as to form a hydrogen bond to Ser48, as was suggested from the model building of Freudenreich et al. (1984). With this positioning, the methylene or methyl group α to the carbonyl carbon interacts too closely (about 2.6 Å) with the benzene ring of Phe93. It is not clear how the enzyme accommodates these ligands without local conformational changes. Substitution of Phe93 with a smaller residue should relieve the steric hindrance. This explanation was tested by studying the horse liver alcohol dehydrogenase with substitutions of two amino acids in the active site, Ser48Thr/Phe93Ala. This enzyme resembles the natural α-isoenzymes found in humans and monkeys, which have high activity on cyclohexanol, a ligand similar in size to δ-valerolactam (Stone et al., 1989; Light et al., 1992). The modeling suggested that the Ser48Thr substitution would not be critical, and the double mutant was of interest as a model for developing specific inhibitors of isoenzymes of alcohol dehydrogenase.

The mutated enzyme was significantly more sensitive to inhibition by the sterically hindered amides, in particular δ-valerolactam (Table 2). Removal of the benzene ring of Phe93 apparently provides additional space for accommodating substituents that are near the oxygen of the ligand. The somewhat weaker affinity of the mutated enzyme for the *N*-monosubstituted formamides reflects the summation of the loss and gain of favorable hydrophobic interactions. This study illustrates an approach toward the rational design of inhibitors with selectivity for particular isoenzymes and provides data for further quantitative analyses.

Since *N*-formylpiperidine was a good inhibitor, kinetic parameters for the analogous substrate, cyclohexanecarboxaldehyde, and the reduced form, cyclohexylmethanol, were determined (Table 3). The catalytic efficiency on the alcohol

Table 3: Kinetic Constants for Reactions of Cyclohexylmethanol and Cyclohexanecarboxaldehyde with Horse Liver Alcohol Dehydrogenase[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
cyclohexylmethanol	540 ± 100	3.1 ± 0.3	5.7
cyclohexanecarboxaldehyde	15 ± 1	54 ± 1	3700

[a] Reactions were studied at pH 8 and 25 °C with 2 mM NAD^+ and 0.2−1.0 mM alcohol or 0.1 mM NADH and 0.011−0.1 mM aldehyde.

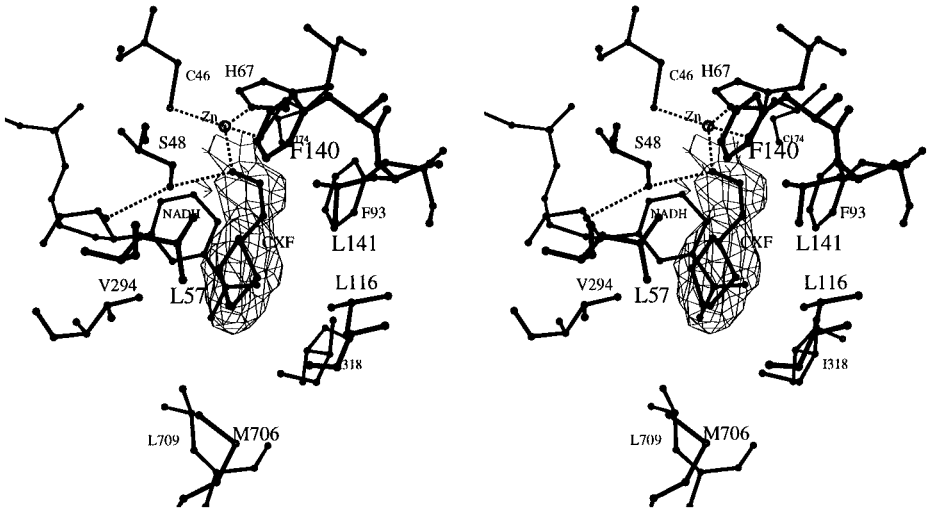

FIGURE 1: Active site of horse liver alcohol dehydrogenase complexed with NADH and *N*-cyclohexylformamide (CXF). An electron density map ($2|F_o| - |F_c|$) was calculated at one standard deviation above the mean including the atoms of the inhibitor with the refined structure. The dashed lines indicate the coordination of the catalytic zinc and the hydrogen bonds of the proton relay system. M706 and L709 are Met306 and Leu309 from the adjoining subunit, respectively.

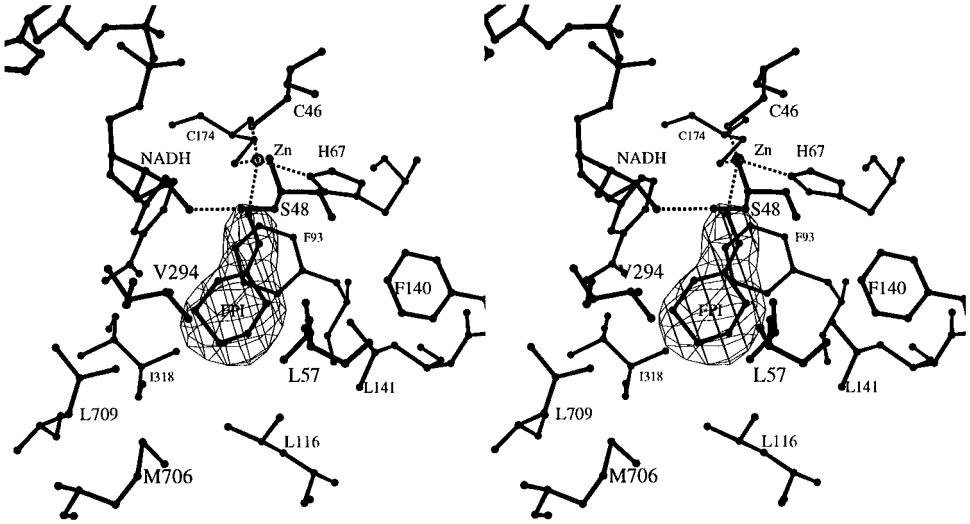

FIGURE 2: Active site of horse liver alcohol dehydrogenase complexed with NADH and *N*-formylpiperidine (FPI). A difference electron density map ($|F_o| - |F_c|$) was calculated at one standard deviation above the mean without including the atoms of the inhibitor.

was comparable to the activity of the enzyme on primary and branched-chain alcohols (Light et al., 1992), and efficiency with the aldehyde was 4-fold higher than with benzaldehyde (Shearer et al., 1993). These results suggest that the enzyme readily recognizes this structure and that the corresponding formamide could bind in a mode that resembles the Michaelis complex.

X-ray Crystallography. Although docking of ligands into the active site may produce reasonable models, actual structures should be determined. Thus, structures of horse liver alcohol dehydrogenase complexed with NADH and *N*-cyclohexylformamide or *N*-formylpiperidine were solved at 2.5 Å resolution. All four subunits in the unit cell have very similar structures, which are in the closed conformation found for other ternary complexes, such as the one with NAD^+ and pentafluorobenzyl alcohol (Ramaswamy et al., 1994). Although the interactions of the subunits in the crystal lattice differ, the peptide backbone is not affected. Some side chains have alternate positions, but these will not be defined at the present resolution.

The locations of the amides were well-defined in the electron density maps (Figures 1 and 2). The amide group of *N*-cyclohexylformamide is *cis*, as the *trans* isomer would not fit into the density. The amide of *N*-formylpiperidine is also planar. The interactions in the complexes are summarized in Table 4. Both compounds bind with their oxygens ligated to the catalytic zinc and hydrogen bonded to the hydroxyl group of Ser48. The carbonyl groups are positioned similarly, but the rings of the two compounds are oriented differently, being almost perpendicular to one another (Figure 3). The side chains of most amino acid residues are in similar positions in the two complexes, but Leu116 and Met706 (Met306 of the other subunit) have different conformations, which reflect optimal interactions with the formamides.

The hydrogen-bonded network that links the O of the inhibitor to His51 via the 2′-hydroxyl group of the nicotinamide ribose is intact (Ramaswamy et al., 1994). This system appears to relay a proton between the substrate and His51, which acts as an acid/base catalyst. The distance of 3.9 Å between the nitrogen of *N*-cyclohexylformamide and the oxygen of the hydroxyl group of Ser48 is not consistent with a hydrogen bond between these atoms, as was proposed on the basis of model building (Freudenreich et al., 1984).

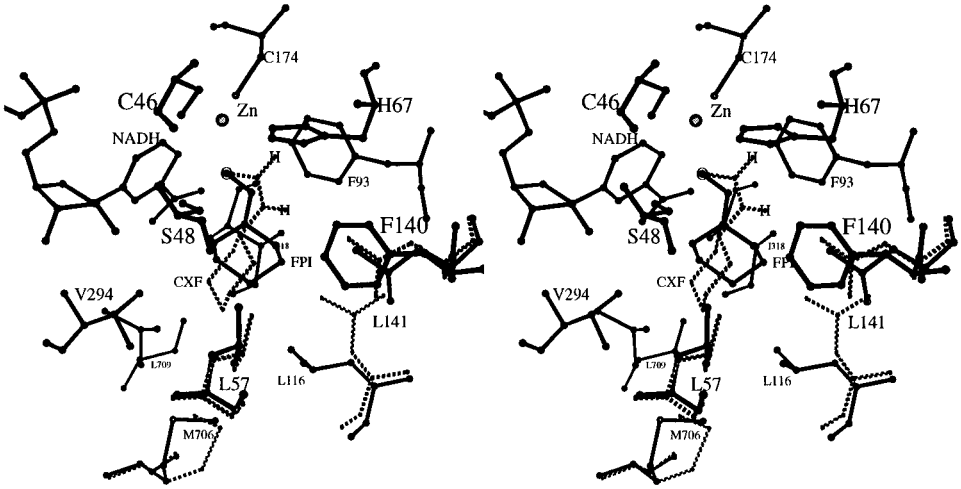

FIGURE 3: Comparison of the binding modes of *N*-formylpiperidine (FPI) and *N*-cyclohexylformamide (CXF, dotted lines). The atoms for most of the residues of the protein and coenzyme (NADH) have very similar coordinates in both complexes, but the differences are illustrated with Leu57, Leu116, Leu141, and Met706 (Met306 from the adjoining subunit). The hydrogen atoms of *N*-cyclohexylformamide are labeled in order to indicate the proximity to Phe93.

Table 4: Interactions of Formamides with the Enzyme−NADH Complex[a]

N-cyclohexylformamide		Å	*N*-formylpiperidine		Å
O	Zn	2.3	O	Zn	2.3
O	OG Ser48	2.6	O	OG Ser48	2.5
O	C5N NADH	3.7	O	C5N NADH	3.2
C	C4N NADH	4.0	C	C4N NADH	4.0
N	OG Ser48	3.9	N	OG Ser48	3.5
N	CE1 Phe93	3.0	N	CE1 Phe93	4.1
C1	C4N NADH	4.2	N	C4N NADH	4.2
C2	O7N NADH	3.5	C2	OG Ser48	3.3
C3	CG2 Val294	3.8	C3	C7N NADH	3.5
C4	CD2 Leu116	3.9	C4	CD2 Leu116	3.3
C5	CD2 Leu57	3.6	C5	CE1 Phe93	3.8
C6	CZ Phe140	5.3	C6	CD1 Leu141	3.5

[a] Distance in angstroms units between atoms of the formamides and the closest atoms of the protein or NADH.

Instead, the amide NH interacts at a distance of 3.0 Å with the benzene ring of Phe93 (Figure 1). This contact resembles the "amino−aromatic" or "cation-π" interactions described for a variety of structures (Burley & Petsko, 1988; Dougherty, 1994). With *N*-formylpiperidine, this distance is 4.1 Å, probably too long for a significant interaction.

The distance between the carbonyl carbons of *N*-cyclohexylformamide and *N*-formylpiperidine and C4N of NADH is 4.0 Å. This distance is about the same as that found in the complex with H_2NADH and *p*-(*N*,*N*-dimethylamino)-cinnamaldehyde (Cedergren-Zeppezauer et al., 1982) or between C4N of NAD^+ and the methylene C of *p*-bromobenzyl alcohol, but longer than the 3.4 Å in the complex with NAD^+ and pentafluorobenzyl alcohol (Ramaswamy et al., 1994). These distances are in the range where a direct hydride transfer would be possible, considering that the enzyme is a dynamic molecule with random motions of 0.5 Å. There appear to be no steric conflicts that would prevent the formamide groups from approaching C4 of the nicotinamide ring to within a distance of about 3.5 Å, which would approximate the ground state for hydride transfer (Tapia et al., 1991; Olson et al., 1996). At the transition state, the C−C distance is calculated to be 2.6−2.9 Å (Tapia et al., 1988; Wu et al., 1995; Olson et al., 1996). Such a close approach is consistent with the isotopic results that indicate that the reaction catalyzed by alcohol dehydrogenase proceeds with hydrogen tunneling (Bahnson et al., 1993).

Significantly, the carbonyl groups of the formamides are oriented so that, if hydride ion transfer were to occur, it would be to the *re* face and would produce a product that resembles the complex with NAD^+ and pentafluorobenzyl alcohol (Ramaswamy et al., 1994). Thus, the geometry of the interactions suggests that the formamides mimic the Michaelis complex of NADH and aldehyde. *N*-Formylpiperidine can be regarded as a nonreactive analog of cyclohexylcarboxaldehyde or benzaldehyde. In all of these observed complexes, the O of the ligand is very close to C5 of the nicotinamde ring so that the C═O bond is almost parallel with the C4−C5 bond. The orientation of the carbonyl group is rotated about 60° from the position suggested from a structure−function study with chiral amides (Sharma & Woronick, 1971).

The close interaction of the O with C5 of the nicotinamide ring may be relevant for activation of the coenzyme and for binding of the O of the ligand. 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 (Almarsson & Bruice, 1993; Wu et al., 1995). The resolution of the present three-dimensional structures is not sufficient to detect ring puckering, but resonance Raman spectra of specifically deuterated NADH bound to lactate or malate dehydrogenases offer experimental support (Deng et al., 1992). The O of the ligand is not within hydrogen-bonding distance of the C2H of His67, as was suggested on the basis of molecular dynamics simulations (Olson et al., 1996).

Structural Correlations. In contrast to the binding observed with the formamides, the complex with *p*-(*N*,*N*-dimethylamino)cinnamaldehyde does not have the *re* face oriented toward the nicotinamide ring. However, a simple rotation of 30−40° would give an orientation and a distance of about 3.6 Å that appear to be suitable for direct hydride ion transfer. In this regard, the observed position of the aldehyde is similar to that found for *p*-bromobenzyl alcohol in the complex with NAD^+, in that a small rotation would be required to orient the *pro-R* hydrogen toward the nicotinamide ring, giving the position found in the complex with pentafluorobenzyl alcohol (Ramaswamy et al., 1994).

The differences in energies of these positions are probably not very large, since no steric barriers are obvious from the structures, and the rotations should be fast relative to the chemical steps.

The structures of liver alcohol dehydrogenase complexed with NADH and dimethyl sulfoxide or 3-butylthiolane 1-oxides also have a geometry that does not seem to mimic closely a ground state poised for reaction (Al-Karadaghi et al., 1994; Cho et al., 1997). The distance from the S to C4N of NADH is about 3.7 Å, and the sulfoxide is rotated about 90° away from a position that would seem optimal for direct hydride transfer to the analogous keto compound. Indeed, the S is about 3.4 Å from the benzene ring of Phe93, in what appears to be a cation−π interaction.

The structures of several complexes illustrate the fact that subtle interactions determine the various binding modes and make it difficult to predict how a ligand will bind. The enzyme is flexible and can accommodate ligands in different ways. Knowing the structures of the formamides provides a better basis for the rational design of selective inhibitors and for dynamic simulations of the hydrogen transfer reactions.

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