Structure of the Liver Alcohol Dehydrogenase–NAD^+–Pyrazole Complex As Determined by ^{15}N NMR Spectroscopy†

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ABSTRACT: The structures of the liver alcohol dehydrogenase (LADH)–NAD^+–pyrazole and LADH–NAD^+–4-ethylpyrazole complexes were investigated by ^{15}N nuclear magnetic resonance (NMR) spectroscopy. ^{15}N chemical shifts were obtained for ^{15}N-labeled inhibitors and ^{15}N-labeled coenzyme bound in the ternary enzyme complexes. The structures of the two inhibitor complexes appear to be very similar. ^{15}N NMR studies of model pyrazole–zinc chloride complexes were carried out to determine the effect of zinc complexation on pyrazole chemical shifts. The N1 nicotinamide chemical shift of the coenzyme of the LADH–NAD^+–pyrazole complex demonstrates that NAD^+ is converted to a dihydronicotinamide derivative in the complex. The N1 chemical shift of the pyrazole in the ternary complex is consistent with covalent bond formation between pyrazole N1 and the nicotinamide ring of the coenzyme. The N2 chemical shift of the pyrazole in the ternary complex indicates that the nucleus of this nitrogen is about 40 ppm more shielded than those of the N2 nitrogens of typical pyrazoles. Such shielding is expected as the result of direct complexation of N2 to the active-site zinc. Shift comparisons with zinc–pyrazole complexes indicate a high degree of inner-sphere coordination of the pyrazole N2 to the active-site zinc in the ternary complex.

The structure of inhibitor complexes of alcohol dehydrogenase from horse liver (LADH) has been the subject of many recent studies. LADH is a zinc-containing dimer of $M_r \sim 80000$ which catalyzes the NAD^+-dependent, reversible oxidation of ethanol to ethanal (Brändén et al., 1975; Klinman, 1981). The active site has a nonspecific substrate-binding region capable of binding a variety of substrates and inhibitors in close proximity to both the catalytic zinc atom and nicotinamide ring of the coenzyme. The role of the active-site zinc, both in catalysis and in binding of substrates and inhibitors, has been of great interest. X-ray crystallographic studies support direct binding of substrate or inhibitor to the catalytic zinc in crystalline complexes (Boiwe & Brändén, 1977; Plapp et al., 1978; Eklund et al., 1981, 1982a,b; Cedergren-Zeppezauer et al., 1982). Other studies of metal-substituted enzymes in solution have been interpreted as supporting second-sphere coordination of substrate or inhibitor to the active-site metal (Sloan et al., 1975; Drysdale & Hollis, 1980; Bobsein & Myers, 1981). It is always possible that the structure of a crystalline enzyme complex is not necessarily representative of the structure in solution. Indeed, this question is raised for LADH by its formation of a nonproductive crystalline ternary complex with *p*-bromobenzyl alcohol (Eklund et al., 1982a). It is therefore important to be able to investigate the structures of ternary complexes of LADH as they exist in solution.

Pyrazole is a potent inhibitor of LADH. The structure of the LADH–NAD^+–pyrazole complex is of interest not only because of possible implications for the catalytic mechanism of LADH but also because of its potential medical importance in the treatment of alcoholism, as well as methanol and ethylene glycol poisoning (Deis & Lester, 1979). In the presence of NAD^+, pyrazole forms a tight ternary complex with LADH which has a K_I of 0.2 μM (Theorell et al., 1969) and a lifetime of 25 s at pH 7 (Shore & Gilleland, 1970). A less toxic derivative of pyrazole, 4-ethylpyrazole, is an even stronger inhibitor of LADH, with K_I of 0.007 μM in the presence of NAD^+ (Dahlbom et al., 1974). Theorell & Yonetani (1963) first proposed on the basis of spectrophotometric evidence that N1 of pyrazole forms a covalent bond to C4 of the nicotinamide ring of the coenzyme in the ternary complex. They also proposed that N2 of the pyrazole derivative is directly coordinated to the active-site zinc. This structure has recently been given strong support by the X-ray studies of Eklund and co-workers (Eklund et al., 1982b) for crystalline LADH–NAD^+–pyrazole complexes. The pyrazole N1–nicotinamide C4 bond distance was determined to be 2 Å and the pyrazole N2–active-site zinc atom distance, 2.1 Å. However, Bobsein and Myers have interpreted ^{113}Cd chemical shifts of the cadmium-substituted LADH–NAD^+–pyrazole complex as demonstrating outer-sphere coordination of the inhibitor to the active-site zinc (Bobsein & Myers, 1981).

In this paper, we report the use of ^{15}N NMR spectroscopy to investigate the structure of the LADH–NAD^+–pyrazole complex in solution. ^{15}N NMR spectroscopy is a sensitive tool for the determination of the oxidation state of the coenzyme NAD(H) (Oppenheimer & Davidson, 1980) and of the structure of pyrazole derivatives (Schuster et al., 1979) as well as the extents of hydrogen bonding and metal complexation, especially for azine (=N̈–) nitrogens. However, ^{15}N NMR spectra of large biomolecules are often difficult to obtain because of the low nuclear moment of ^{15}N (about one-tenth that of ^1H) and the line broadening of resonances associated with long correlation times. In the present study, we have obtained ^{15}N NMR spectra from ternary enzyme complexes prepared from doubly ^{15}N-labeled pyrazole, doubly ^{15}N-labeled 4-ethylpyrazole, and N1-^{15}N-labeled NAD^+.

Materials and Methods

All reagents used were of reagent-grade purity unless otherwise specified. [$^{15}N_2$]Pyrazole (99% isotopic abundance) was prepared from [$^{15}N_2$]hydrazine sulfate (Stohler, 99% isotopic abundance) and 1,1,3,3-tetraethoxypropane (Aldrich) by the method of Jones (1949) and subsequently purified by sublimation. 4-Ethyl[$^{15}N_2$]pyrazole was prepared in a similar manner by using 1,1,3,3-tetraethoxy-2-ethylpropane prepared (Klimko & Skoldinov, 1959a,b) from 1-ethoxy-1-butene (Breitmaier & Gassenmann, 1971). [1-^{15}N]NAD^+ was prepared from [1-^{15}N]nicotinamide (90% isotopic abundance) by

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the method of Oppenheimer & Davidson (1980). The *N*-methylpyrazole–zinc chloride adduct was obtained through addition of 1 molar equiv of zinc chloride to a 1.3 M aqueous solution of *N*-methylpyrazole at pH 6. The resulting precipitate was dried and taken up in chloroform, and, after removal of solvent, the residue had a melting point of 102–108 °C. Anal. Calcd for $C_8H_{12}N_4Cl_2Zn$: C, 31.99; H, 4.03; Cl, 23.60; N, 18.65; Zn, 21.74. Found: C, 31.56; H, 4.21; Cl, 24.23; N, 17.81; Zn, 22.35. Ethyl 2-(1-pyrazolyl)ethanoate was prepared from pyrazole and ethyl 2-bromoethanoate by the method of Jones (1949). Zinc chloride ("Ultrapure", >99% purity) was purchased from Alfa. *N*-Benzylnicotinamidinium chloride was prepared according to the method of Karrer & Stare (1937).

The model pyrazole–nicotinamide adduct was prepared at room temperature by the addition of a 2.0 M solution of sodium hydroxide to an equimolar aqueous solution of pyrazole and *N*-benzylnicotinamidinium chloride, as described by Angelis (1980). The resultant yellow oil was extracted into chloroform and maintained at 0–8 °C to retard decomposition.

LADH was purchased from Boehringer-Mannheim and dialyzed at 4 °C against 0.1 M phosphate buffer, pH 7.0, to remove residual ethanol. After addition, first, of pyrazole and, subsequently, of NAD$^+$ (10- and 15-fold excesses, respectively), the solutions were concentrated by ultracentrifugation at 4 °C. The LADH–NAD$^+$–4-ethylpyrazole complex was prepared from undialyzed enzyme. ^{15}N spectra of the ternary complex, prepared from ^{15}N-labeled pyrazole and unlabeled NAD$^+$, were obtained with a Bruker WH-180 spectrometer operating at 18.25 mHz. Samples were contained in 25-mm tubes and were 1.1 mM (2.2 mN) in enzyme. The temperature for some samples was maintained at 12–14 °C: others were at ambient temperature. Inverse-gated, proton-decoupled spectra were obtained over about 24 h, by using 90° pulses and delays of 1.5–2 s. The ^{15}N spectra of the ternary complex, prepared from both ^{15}N-labeled pyrazole and ^{15}N-labeled NAD$^+$, were obtained with a Bruker WM-500 spectrometer operating at 50.68 MHz. The sample was contained in a 10-mm tube and was 1.5 mM (3 mN) in enzyme, and temperature was maintained at 4 °C. Inverse-gated, proton-decoupled spectra were obtained in about 12 h, by using 90° pulses and 5-s delays to avoid overheating. The ^{15}N spectrum of the ternary complex containing ^{15}N-labeled 4-ethylpyrazole and unlabeled NAD$^+$ was obtained with a Bruker WM-500 spectrometer at ambient temperature. The sample was contained in a 10-mm tube and was 1 mM (2 mN) in concentration. An inverse-gated, proton-decoupled spectrum was obtained in 4 h, by using 90° pulses and 2-s delays. ^{15}N spectra of the pyrazole derivatives were obtained with Bruker WH-180, Bruker WM-500, or Varian XL-200 spectrometers, by using inverse-gated proton decoupling, 45° pulses, and delays of 15 s. All ^{15}N chemical shifts are reported in ppm upfield from external 1 M nitric acid in D_2O.

Results

^{15}N NMR spectra obtained at 18.25 MHz from the LADH–NAD$^+$–pyrazole complex prepared from doubly ^{15}N-labeled pyrazole are illustrated in Figure 1. The observed ^{15}N–^{15}N one-bond coupling constant for the two nonequivalent pyrazole nitrogens, N1 and N2, of the complex was 10 ± 2 Hz. This coupling is similar to those reported for other N-substituted pyrazoles (Hawkes et al., 1977). The line widths for the N1 and N2 doublets were approximately 16 and 20 Hz, respectively.

The sample of the ternary complex prepared from both doubly ^{15}N-labeled pyrazole and ^{15}N-labeled NAD$^+$ gave the

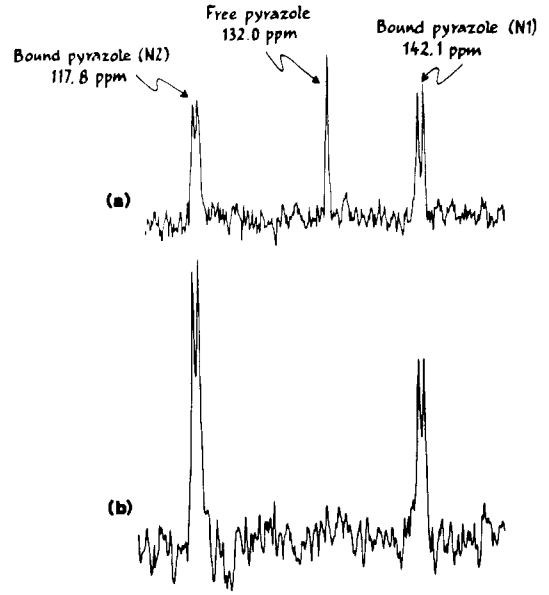


FIGURE 1: ^{15}N spectra at 18.25 MHz of the LADH–NAD$^+$–pyrazole complex prepared from ^{15}N-labeled pyrazole: (a) before dialysis; (b) after dialysis.

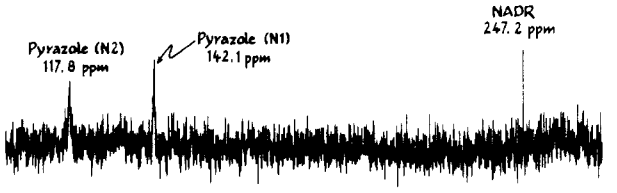
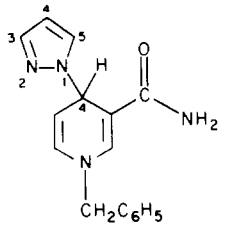


FIGURE 2: ^{15}N spectrum at 50.68 MHz of the LADH–NAD$^+$–pyrazole complex prepared from ^{15}N-labeled pyrazole and ^{15}N-labeled NAD$^+$, after dialysis.

50.68-MHz spectrum shown in Figure 2. The chemical shifts of the pyrazole nitrogens were identical with those found at 18.25 MHz. However, at the higher field, the ^{15}N–^{15}N coupling of the pyrazole nitrogens was unresolved, N1 and N2 each being broadened to about 25 and 30 Hz, respectively, possibly because of increases in the relaxation rate associated with chemical-shift anisotropy or because of chemical exchange. The chemical shift of the nicotinamide ring nitrogen (N1) of the coenzyme in the ternary complex is 96 ppm upfield from that of NAD$^+$ in solution (Oppenheimer & Davidson, 1980), indicating dihydronicotinamide formation, as will be discussed later. The observed line width for this nicotinamide nitrogen resonance was about 5 Hz.

The sample of the LADH–NAD$^+$–4-ethylpyrazole complex prepared from doubly ^{15}N-labeled 4-ethylpyrazole and unlabeled NAD$^+$ gave a ^{15}N spectrum at 50.68 MHz with chemical shifts for the bound pyrazole nitrogens N1 and N2 of 146.0 and 119.0 ppm, respectively, and a chemical shift for the unbound 4-ethylpyrazole of 133.6 ppm (see Table I). As in the 50.68-MHz spectrum of the ternary pyrazole complex, the ^{15}N–^{15}N one-bond coupling was not resolved, the line widths for the N1 and N2 resonances being 25 and 40 Hz, respectively.

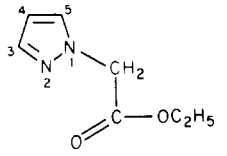
For comparison purposes, we have determined the ^{15}N chemical shifts of various pyrazole derivatives (Table I). Pyrazole itself in aqueous solution gives rise to a single ^{15}N resonance as the result of tautomeric equilibration. The pyrazole–nicotinamide adduct (I) was prepared by addition of base to a mixture of pyrazole and *N*-benzylnicotinamidinium chloride to provide a model for the pyrazole–NAD$^+$ adduct. This adduct has been reported by Angelis (1980) to have a half-life of about 1 h at room temperature. We observed little



I

decomposition at 7–8 °C during the 10 h required to obtain a natural-abundance spectrum.

The effects of complexation with zinc chloride on the chemical shifts of the nitrogens of pyrazole and some of its derivatives are summarized in Table II. The *N*-methylpyrazole–zinc chloride adduct has the empirical formula (*N*-methylpyrazole)$_2$ZnCl$_2$ as determined by elemental analysis. Ethyl 2-(1-pyrazolyl)ethanoate (II) was chosen as one model



II

for the enzyme–inhibitor complex because it was expected that the ester carbonyl group would enhance complexation of zinc chloride to N2 of the pyrazole ring. Other pyrazole derivatives were investigated but either showed reduced complexation as exemplified by smaller changes in the chemical shift or produced zinc complexes of too low solubility for ^{15}N NMR spectra to be obtained.

Discussion

The chemical shift of the nicotinamide ring nitrogen of the coenzyme in the ternary complex, 247.2 ppm, demonstrates unequivocally that the coenzyme in the LADH–NAD$^+$–pyrazole complex has the dihydro structure. The upfield shift resulting from complex formation was +96 ppm relative to free NAD$^+$ and is comparable to the upfield shift in solution, +109 ppm, reported for reduction of NAD$^+$ to NADH (Oppenheimer & Davidson, 1980). The 13-ppm difference most likely reflects the effect of changing the substituent at C4 of the nicotinamide ring from hydrogen to pyrazole, as well as steric and electronic effects of active-site residues of the enzyme.

The chemical shift of the pyrrole-type nitrogen, N1, of pyrazole in the ternary complex, 142.1 ppm, is indicative of covalent-bond formation as the result of addition of pyrazole to the nicotinamide ring of NAD$^+$. The chemical shift of N1 of pyrazole derivatives depends considerably on the nature of the substituent (Table I). Most pyrazole derivatives have N1 shifts greater than 150 ppm. The only pyrazole we have studied that has an N1 chemical shift close to that found for the ternary complex is the pyrazole–*N*-benzylnicotinamide adduct (I), which serves as a model for the pyrazole–NAD$^+$ adduct.[1] Angelis (1980) has reported that, in the adduct, the pyrazole is bonded to nicotinamide at C4. The NMR shifts of our ternary complex cannot be used to assign the position at which pyrazole binds to the nicotinamide ring, but X-ray crystallographic studies also suggest bond formation between N1 of pyrazole and C4 of the nicotinamide ring (Eklund et al., 1982b). The N1 chemical shift found for the enzyme complex in solution is 5.8 ppm upfield from the 136.3-ppm value of the model adduct. This difference could reflect the change in substituent at the N1 nicotinamide nitrogen and/or the effect of interaction with enzyme residues. With regard to the latter, Eklund and co-workers (Eklund et al., 1982b) report a pyrazole N1–C4 nicotinamide ring bond angle of 90° and N1–C4 bond distance of 2 Å, both of which differ from what would be expected for the model adduct.

The pyridine-type nitrogen, N2, of pyrazole in the ternary complex has a chemical shift of 117.8 ppm. This is more than 40 ppm upfield from the N2 chemical shifts of other pyrazoles in the absence of hydrogen bonding (Schuster et al., 1979) and 41.1 ppm upfield from that of the pyrazole–*N*-benzylnicotinamidinium adduct (Table I). The maximum upfield shift that could reasonably be ascribed to hydrogen bonding is about 18 ppm, on the basis of a change in shift of +17.9 ppm for N2 of *N*-methylpyrazole upon change of solvent from chloroform to trifluoroethanol (Schuster et al., 1979). Consequently, the only reasonable explanation for the more than 40-ppm upfield shift of the pyrazole N2 in the ternary complex relative to the model adduct is complexation to the active-site zinc. This conclusion is buttressed by the shift changes of *N*-methylpyrazole and ethyl 2-(1-pyrazolyl)ethanoate upon zinc complexation, 50.8 and 54.9 ppm upfield, respectively, for the N2 resonances in chloroform and acetonitrile (Table II). These shifts may, in fact, be the averaged N2 shifts of complexed and uncomplexed pyrazole derivatives; however, it is expected that pyrazole–metal complexes are only slightly dissociated in acetonitrile and even less so in chloroform (Ährland & Björk, 1975; Daugherty & Swisher, 1968; Reedijk, 1970).

While it is possible that the upfield shift upon zinc complexation of a pyrazole N2 might be expected to be comparable to the protonation shifts of such nitrogens, on the order of 100 ppm (Schuster et al., 1979), the zinc complexation shift is actually about half that. An analogous result has been reported by Alei and co-workers (Alei et al., 1981) for zinc complexation of *N*-methylimidazole, where the zinc complexation shift of N2 is +35–40 ppm and the protonation shift is +73 ppm.

The magnitude of the zinc complexation shift should also be affected by the nature of the other ligands in relation to zinc in the complex. In the enzyme, there are one histidine and two cysteine ligands bound to the catalytic zinc ion, which leaves a fourth coordination site open for complexation of solvent, substrate, or inhibitor. Among the model complexes, the second pyrazole in the *N*-methylpyrazole–zinc chloride adduct could be taken as an analogue for the histidine residue, and chloride ion, a strongly associated ligand in nonnucleophilic solvents such as chloroform, might be an analogue for the cysteine residues. With the ethyl 2-(1-pyrazolyl)ethanoate–zinc chloride complex dissolved in acetonitrile, the ester carbonyl group might serve as an analogue for histidine, with possible competition between chloride ion and acetonitrile for the third and fourth ligand sites. In any case, the zinc complexation shift seems to be on the order of 51–55 ppm with respect to the N2 shift of pyrazole in chloroform or acetonitrile.

The line widths for the nitrogen resonances observed for the LADH–NAD$^+$–pyrazole complex are surprisingly narrow and indicate a high degree of mobility of the pyrazole and nicotinamide moieties. It is possible that some, if not all, of this mobility could arise from a fast exchange between a state where N2 of pyrazole is directly coordinated to zinc and one where water or hydroxide ion is complexed to zinc and hy-

[1] Product mixtures from other pyrazole–nicotinamidinium ion reactions contained uncharacterized compounds with nitrogen resonances close to those of the model adduct I. These products, too unstable for isolation, may be formed by addition of pyrazole at C2 or C6 of the nicotinamide ring or by polymerization of the dihydro derivative.

Table I: ^{15}N Chemical Shifts[a] of Derivatives of Pyrazole

solute	concn (M)	solvent	δ_{15N} N1	δ_{15N} N2	$\bar{\delta}_{15N}$[b]
pyrazole[c]	10×10^{-3}	buffer[d]			132.0
pyrazole	5	CH_3CN			125.5
	4	$(CH_3)_2SO$	166.9[e]	73.9[e]	120.4[e]
4-ethylpyrazole[c]	10×10^{-3}	buffer[d]			133.6
N-methylpyrazole	2	$CHCl_3$	174.6[e]	70.3[e]	122.4[e]
ethyl 2-(1-pyrazolyl)ethanoate (II)	2	CH_3CN	173.3	66.4	119.85
N-benzylpyrazole	2	CH_3CN	161.5	67.3	114.4
3-methyl-*N*-carboxamidopyrazole	3	$(CH_3)_2SO$	152.6	81.2	116.9
LADH–NAD$^+$–pyrazole[c]	1.1×10^{-3}	buffer[d]	142.1	117.8	129.95
LADH–NAD$^+$–4-ethylpyrazole[c]	1×10^{-3}	buffer[d]	146.0	119.0	132.5
pyrazole–nicotinamide adduct (I)	~2	$CHCl_3$	136.3	76.7	106.5

[a] In ppm upfield from external 1 M nitric acid in D_2O. [b] Average shifts of nitrogens. [c] ^{15}N labeled. [d] In 0.1 M sodium phosphate, pH 7.0. [e] From Schuster et al. (1979).

Table II: ^{15}N Chemical Shifts[a] of Zinc Complexes of Pyrazole Derivatives

solute	ratio[c]	solvent	concn[d] (M)	δ_{15N} N1	δ_{15N} N2	$\Delta\delta_{15N}$[b] N1	$\Delta\delta_{15N}$[b] N2
pyrazole–$ZnCl_2$	2:1	CH_3CN	3	148.2			(+44)[e]
	1:1	$(CH_3)_2SO$	1	138.1			(+34)[f]
(*N*-methylpyrazole)$_2ZnCl_2$	2:1	$CHCl_3$	0.4	175.9	121.1	+1.3	+50.8
ethyl 2-(1-pyrazolyl)ethanoate–$ZnCl_2$	2:1.5	CH_3CN	2	175.6	121.3	+2.3	+54.9

[a] In ppm upfield from external 1 M nitric acid in D_2O. [b] Change in chemical shift; cf. uncomplexed compound (Table I). [c] Molar ratio of pyrazole derivative to zinc chloride. [d] Concentration of pyrazole derivative. [e] Approximate change in shift, based on a change in the average shift of +22.7 ppm. [f] Approximate change in shift, based on a change in the average shift of +17.7 ppm.

drogen bonded to N2 of pyrazole, in effect, an inner-sphere–outer-sphere equilibration process. Such exchange could lead to line broadening as the strength of the magnetic field is increased and, together with line broadening from chemical-shift anisotropy, account for the greater line widths observed at 50.68 MHz compared to 18.25 MHz. If one expects *full* zinc complexation of N2 to give a resonance at 121 ppm, as in the zinc complexes of *N*-methylpyrazole and of II (Table II), and the shift of a fully hydrogen-bonded N2 to be ~95 ppm (18 + 76.7 ppm, the N2 shift of I in chloroform), then the observed 118-ppm resonance position for N2 corresponds to a very high degree of inner-sphere coordination. Indeed, second-sphere coordination of pyrazole to zinc in the ternary complex could well be totally absent, and, if so, the unexpected sharpness of the resonances has to be accounted for by other forms of mobility at or near where the pyrazole–NAD$^+$ moiety is connected to the enzyme. In the crystalline complex, there seems to be sufficient room in the substrate binding pocket for the pyrazole nucleus to rotate ±20° around the N2–zinc axis (Eklund et al., 1982b), but it is not clear whether this is enough to give the sharp ^{15}N resonances in solution. Another possibility is a rather loose and mobile coordination of the nucleotide end of the complex, and this actually agrees well with the specially narrow line width of the N1 resonance of the nicotinamide group. Studies of the cadmium-substituted enzyme–NAD$^+$–pyrazole complex indicate that it has a predominantly outer-sphere metal to pyrazole coordination (Bobsein & Myers, 1981), and this could account for the lesser catalytic activity of the cadmium enzyme.

The LADH–NAD$^+$–4-ethylpyrazole complex was prepared to determine what effect tighter binding of inhibitor has on the N1 and N2 resonances of the pyrazole nucleus. The inhibition constant in the presence of NAD$^+$ of 4-ethylpyrazole is almost 30 times smaller than that of pyrazole (K_I = 0.007 and 0.2 μM, respectively). Alkyl substituents at the 4-position appear to increase the stability of pyrazole inhibitor complexes through interactions between the substituent and the hydrophobic cleft of the substrate-binding region of the enzyme (Winer, 1977; Eklund et al., 1982b). While the ternary complex containing 4-iodopyrazole [K_I = 0.02 μM (Dahlbom et al., 1974)] was found by X-ray crystallography to have a structure which is very similar to that of the complex containing pyrazole itself, these hydrophobic interactions may lead to a solution conformation of the pyrazole nucleus in the 4-ethylpyrazole complex which differs from that of the pyrazole complex. The pyrazole N1 chemical shift of the ternary complex might be expected to reflect changes in the N1–C4 bond distance and angle to the nicotinamide ring. The N2 chemical shift should reflect changes in the N2–zinc bond distance and changes in inner-sphere–outer-sphere equilibration, if present. The line widths of the several resonances should reflect restrictions in mobility of the pyrazole nucleus.

The ^{15}N shifts of the LADH–NAD$^+$–4-ethylpyrazole complex indicate that the structure is very similar to that of the LADH–NAD$^+$–pyrazole complex (Table I). The N1 chemical shift in the 4-ethylpyrazole complex, 146.0 ppm, is 3.9 ppm upfield from that of the pyrazole complex, while the N2 chemical shift, 119.0 ppm, is 1.2 ppm upfield from that of the pyrazole complex. These are small changes, but in the direction of somewhat greater zinc complexation. The line widths also indicate tighter binding and somewhat less mobility of the pyrazole nucleus in the 4-ethylpyrazole complex. The N2 line width at 50.68 MHz was 40 Hz, one-third greater than that found for the pyrazole complex, while the N1 line width was about the same in both complexes. These changes would result if, in the 4-ethylpyrazole ternary complex, the mobility of N2 is relatively more restricted, while that of N1 is not. Tighter binding to N2 would make for greater stability of the inhibitor complex and account for the upfield shift of N2 relative to the unsubstituted pyrazole complex.

The sharp ^{15}N lines observed for the LADH–NAD$^+$–pyrazole complex are noteworthy for a complex presumably of molecular weight over 80000. These lines do not arise from formation of low molecular weight substances, because the complex from which they arise is not dialyzed. Clearly, the effective correlation times for the pyrazole and nicotinamide

portions of the inhibitor complex are much shorter than those expected for the bulk of the enzyme, despite the apparent tightness of binding of the inhibitor to the NAD^+ and the catalytic zinc atom. There is an important message here, namely, that prejudgements should be avoided which suggest that the application of NMR to the general investigation of small molecules bound to large biomolecules is bound to give such broad lines as to be fruitless to investigate.

In summary, the LADH–NAD^+–pyrazole complex in solution has chemical shifts for the pyrazole N1 and the nicotinamide ring nitrogens which suggest bond formation between pyrazole N1 and nicotinamide C4, in agreement with the model first proposed by Theorell and Yonetani. The chemical shift of the pyrazole N2 for the ternary complex is indicative of a high degree of inner-sphere coordination to the active-site zinc. While we cannot absolutely exclude partial outer-sphere coordination, the structure of the ternary complex in solution appears to be very similar to that found for the crystalline enzyme complex.

Acknowledgments

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Registry No. I, 90047-63-5; II, 10199-61-8; NADH, 58-68-4; pyrazole, 288-13-1; 4-ethylpyrazole, 17072-38-7; *N*-methylpyrazole, 930-36-9; *N*-benzylpyrazole, 10199-67-4; 3-methyl-*N*-carboxamidopyrazole, 873-50-7; $(pyrazole)_2ZnCl_2$, 64525-82-2; pyrazole–$ZnCl_2$, 90065-12-6; $(N\text{-methylpyrazole})_2ZnCl_2$, 90065-13-7; zinc, 7440-66-6.

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