Properties of Bound Trifluoroethanol Complexes with Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The substrate analogue 2,2,2-trifluoroethanol (TFE) has been used as a ^{19}F NMR probe of the active site of alcohol dehydrogenase from horse liver (LADH). We are able to directly observe a single resonance assigned to TFE in its ternary complex with LADH and nicotinamide adenine dinucleotide (NAD). The chemical shift of this resonance is independent of pH between values of 6.2 and 8.9, suggesting that bound TFE does not change ionization state in this range. Both by ^{19}F NMR self-exchange measurements and by ligand-displacement studies with pyrazole, we also find that displacement of TFE from its ternary complex with NAD is a linear function of proton concentration over a similar pH range, with more rapid desorption occurring at lower pH values. This suggests that the pK of 6.4 for this process seen previously by Kvassman and Pettersson [Kvassman, J., & Pettersson, G. (1980) *Eur. J. Biochem. 103*, 557] is not due to the ionization of bound TFE. These studies also show that the bound lifetime of TFE in its ternary complex with LADH and NAD is quite long (400 s) at pH 8.7, suggesting the use of TFE as a kinetic trapping reagent in single-turnover stopped-flow experiments. Binding isotherms of NAD to LADH saturated with TFE at pH 8.7 or with pyrazole at pH 7.5 reveal essentially no cooperative behavior. The displacement time courses described above are all adequately fit as first-order processes, thus giving no evidence for site heterogeneity or site–site interaction in the binding of these ligands to dimeric LADH. TFE and pyrazole are used as reagents to further explore the question of site–site interaction from a kinetic point of view in the following paper [Anderson, D. C., & Dahlquist, F. W. (1982) *Biochemistry* (following paper in this issue)].

One of the best-studied simple oligomeric enzymes is equine liver alcohol dehydrogenase (LADH). A dimeric zinc metalloenzyme with a subunit molecular weight of 42 000, its chemical mechanism, structure, and the possible existence of intersubunit interactions during catalysis have been extensively studied in recent years (Brändén et al., 1975; Brändén & Eklund, 1980). In spite of considerable work, important aspects of the mechanism remain elusive.

One of the most vigorously disputed mechanistic issues has concerned the existence or degree of ligand-mediated subunit interactions during catalysis [see Weidig et al. (1977) or Kvassman & Pettersson (1978) and Dunn et al. (1979) for opposing viewpoints]. An equally important area concerns the chemistry involved in catalysis of a wide range of alcohol substrates. This includes the role of the active-site zinc ion (Shore et al., 1974; McFarland & Chu, 1975; Dworschack & Plapp, 1977; Plapp et al., 1978; Kvassman & Pettersson, 1978, 1980a,b) and the identities of ionizing groups responsible for the various pK_as which affect the substrate binding thermodynamics and kinetics of this enzyme (Laws & Shore, 1978; Wolfe et al., 1977; Kvassman & Pettersson, 1978, 1980a,b).

Since horse LADH is a fairly nonspecific enzyme, it has been used with a wide variety of primary and secondary alcohol–aldehyde substrate pairs, as well as substrate analogues and competitive inhibitors (Sund & Theorell, 1963). One such inhibitor is trifluoroethanol (TFE), a nonreactive alcohol which forms a tight LADH ternary complex with nicotinamide adenine dinucleotide (NAD) (Sigman, 1967; Dubied & von Wartburg, 1976). Since it is nonreactive, it can be useful in dissecting the enzyme's mechanism on the alcohol side of the central ternary complexes E·NAD·alcohol and E·NADH·aldehyde, without interference from steps beyond or involving hydride transfer. In its ternary complex with LADH and NAD, TFE has been suggested to be complexed to the active-site zinc in a manner similar to *p*-bromobenzyl alcohol in its presumed active ternary complex (Plapp et al., 1978), and has been studied in Co^{2+}-substituted LADH by Drysdale & Hollis (1980).

The trifluoromethyl residue provides a potentially highly sensitive probe of TFE interactions with LADH by virtue of its ^{19}F NMR spectrum. Thus, we have been able to probe the chemical nature of TFE in its ternary complex with LADH and NAD. We find that the chemical shift of the bound alcohol is not suggestive of a covalent zinc–alcohol adduct and is probably not ionized in the pH range 6–10. Our data suggest it is present as the neutral alcohol, with no detectable pK_a between pH 6 and 9.

We are able to examine subunit interactions by obtaining NAD binding isotherms in the presence of TFE or a similar inhibitor, pyrazole. We find essentially hyperbolic NAD binding to E·TFE and E·pyrazole, suggesting no cooperativity in NAD binding for either ternary complex. Combining equilibrium ^{19}F NMR spin–spin relaxation measurements and ligand-displacement studies, we can determine TFE desorption rates over the pH range from 6 to 10. The prominent path for ligand desorption from the E·NAD·TFE ternary complex appears to involve TFE desorbing before NAD, with a very slow single exponential rate. This single desorption rate further suggests equivalent behavior of the two subunits in alcohol desorption.

The slow desorption rates of TFE from its ternary complex at higher pH values, combined with the lack of any significant UV absorbance above 290 nm, suggest the use of TFE as an alternative tightly bound inhibitor to pyrazole (McFarland & Bernhard, 1972) for use in single-turnover kinetic experiments. Applications of this use of both TFE and pyrazole as kinetic traps, and of the ternary complex NAD binding isotherms to further study subunit interactions in LADH, are presented in

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the following (Anderson & Dahlquist, 1982).

Materials and Methods

LADH used in these studies was isolated and purified as previously described by Anderson & Dahlquist (1979). Trifloroethanol and pyrazole (reagent grade) were obtained from Aldrich. Grades III and V NAD and NADH were from Sigma; grade V NAD was used in binding experiments.

For use in ^{19}F NMR experiments, TFE-*1*-d_2 was synthesized to avoid splitting of the fluorine resonance by protons on the adjacent carbon atom. $LiAlD_4$ (0.8 g) was dispersed in 50 mL of anhydrous ethyl ether under nitrogen. Trifluoroacetic acid (2.5 mL) in 10 mL of diethyl ether was slowly added. Refluxing was maintained for 1 h after addition. H_2O (1 mL), 15% NaOH (1 mL), and H_2O (3 mL) were serially added to quench the excess $LiAlD_4$. After filtration of the precipitate, ether was carefully distilled off at 34–36 °C. Proton NMR spectra revealed no peaks, while ^{19}F NMR spectra showed a single resonance.

The concentration of TFE-*1*-d_2 in stock solutions was assayed by ^{19}F NMR by comparison of the integrated peak intensity of a known amount of TFE with the intensity of the ^{19}F resonance of trifluoroacetate (TFA). TFA was determined by titration with a Dilut-it solution of 0.1 M NaOH.

Preparation of Adducts of Diethylzinc and Diphenylzinc with TFE. The covalent monosubstituted adducts of TFE with diphenylzinc (Alfa Products, Inc., Danvers, MA) and diethylzinc were prepared according to Bruce et al. (1966). All glassware and serum caps were oven-dried at 120 °C overnight before use. C_6D_6 (Aldrich) and α,α,α-trifluorotoluene (Aldrich), used as an integral nonreactive chemical shift standard, were dried over 3-Å molecular sieves for several days before use. Typically, 100 mg of diphenylzinc or ca. 1 mL of diethylzinc was added to 3 mL of dry C_6D_6 while rapidly stirring, so that the diphenylzinc or diethylzinc remained in large excess. ^{19}F NMR spectra were then collected, and the shift was compared to a standard of TFE-*1*-d_2 and $C_6H_5CF_3$ in C_6D_6. The C_6D_6 was used as an internal lock in these experiments. NMR spectra were obtained with a Nicolet NT 150 spectrometer operating at 141.2 MHz for fluorine. Spectra of samples containing enzyme were collected without spinning in 12-mm internal diameter tubes containing 10% v/v D_2O for an internal lock. Chemical shifts are reported relative to trifluoroacetate (TFA), which was used for an internal chemical shift and field homogeneity standard for aqueous samples. All reported chemical shifts are downfield from internal trifluoroacetate. No evidence of trifluoroacetate binding to LADH was observed under any experimental conditions at the concentrations used in all experiments, usually ca. 0.2 mM. pH as read on a Copenhagen Radiometer 25 pH meter with a combination electrode was measured before and after NMR experiments at the same temperature as the sample in the probe. The pH is represented as pH*, the apparent pH of the solutions which included 10% v/v D_2O with no corrections. Spin–spin relaxation times (T_2) were determined by the Hahn spin-echo technique. T_{2f} was determined by adding a large excess of pyrazole and NAD to completely displace TFE from the enzyme's active sites. Data sets of L_0, $(1/T_{2,app} - 1/T_{2f})^{-1}$, and E_0, obtained at pH 8.75 and 20 °C, were fitted by a nonlinear least-squares procedure to nonlinearized forms of binding eq 6 and 10.

Fluorescence Measurements. NAD binding to LADH in the presence of varying amounts of TFE or pyrazole, resulting in a quench of protein fluorescence, was determined on a Schoeffel fluorometer. Excitation through tandem monochromators was at 300 nm with a resolution of 32 Å for 2-mm excitation slits. Emission at 340 nm was expressed as a ratio of the emission photomultiplier voltage to excitation photomultiplier voltage. The excitation photomultiplier recorded light intensity by using a rhodamine B standard in ethylene glycol.

Typically, 2–2.5-mL solutions of enzyme and TFE (or pyrazole) in a 3-mL quartz cuvette were allowed to thermally equilibrate for 10–15 min with the thermostated cell holder, which was kept at 25 ± 0.2 °C with an external water bath. Readings of enzyme fluorescence were allowed to stabilize to less than 1% fluctuations before titration commenced. Aliquots of NAD, with enzyme and TFE at the same concentration as in the quartz cuvette to avoid the necessity of correcting for dilution effects, were added with micropipets, taking care not to immerse the pipet tip into the enzyme solution. Computerized measurement routines for the Varian 620i computer were then initialized. The contents were stirred under computer control for about 20 s. A minimum necessary mixing time of 5 s was established for this apparatus by using indicator dyes.

Since the ligand should equilibrate with E·NAD approximately with the sum of the bimolecular on and unimolecular off rates, the solution was allowed to sit for a period exceeding $5k_{off}^{-1}$ for TFE or pyrazole to ensure equilibration. Ten thousand readings of the emission and excitation photomultiplier voltages were then ratioed and averaged, and the next aliquot was added.

A nonlinear least-squares regression analysis for two binding sites and, generally, a nonlinear fluorescence quench, was performed on the data to obtain the best-fit values. For the most general case involving a dimer of concentration E_0, the change in fluorescence (ΔF) was regarded to be a function of two phenomenological Adair constants, ψ_1 and ψ_2, and a fluorescence change for the binding of the first and second ligands, ΔF_1 and ΔF_2. For a linear fluorescence quench upon binding to a dimer, $\Delta F_1 = {}^1/_2\Delta F_2$. The interpretations of ψ_1 and ψ_2 are model dependent [see Mueggler et al. (1975)]. For the general case

$$\Delta F_{obsd} = \frac{\psi_1 x \Delta F_1 + \psi_2 x^2 \Delta F_2}{1 + \psi_1 x + \psi_2 x^2} \quad (1)$$

where x = free ligand concentration. The value of x can be calculated from the total ligand concentration x_0 by the relationship

$$x = x_0 - E_0 \frac{\psi_1 x + 2\psi_2 x^2}{1 + \psi_1 x + \psi_2 x^2} \quad (2)$$

Rearranging gives

$$-\psi_2 x^3 + [(x_0 - 2E_0)\psi_2 - \psi_1]x^2 + [(x_0 - E_0)\psi_1 - 1]x + x_0 = 0 \quad (3)$$

This cubic equation has three roots. Using the real root between x_0 and $x_0 - 2E_0$, we calculated a value for x, the free ligand concentration, for all points of the titration. Using initial estimates of ΔF_1, ΔF_2, ψ_1, and ψ_2, we calculated ΔF by eq 6 for each titration point. VA13A, a subroutine supplied to the University of Oregon by the Harwell Subroutine Library, is used to minimize the quantity $\sum(\Delta F_{calcd} - \Delta F_{obsd})^2$ with respect to the parameters ΔF_1, ΔF_2, ψ_1, and ψ_2. Any combination of up to three parameters may be held constant for the minimization. For example, if fluorescence is known to change linearly with bound ligand, ΔF_1 and ΔF_2 will be fixed at a value determined from the plot of $([NAD]_0 - E_0)^{-1}$ vs. ΔF_{obsd}^{-1}. Minimization will then proceed with respect to ψ_1 and ψ_2.

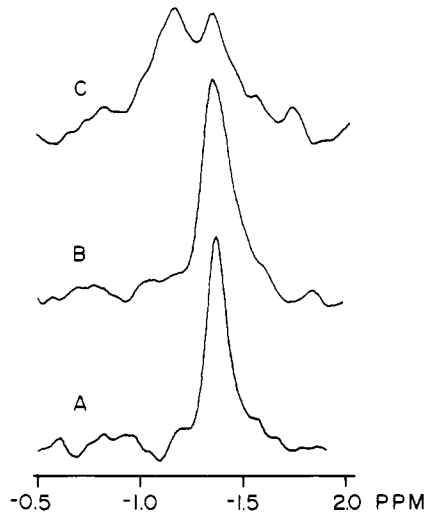


FIGURE 1: ^{19}F NMR spectra of free TFE-*1*-d_2 and binary E·TFE and ternary E·NAD·TFE complexes. In (A), the free TFE resonance occurs 1.36 ppm upfield from the internal standard TFA (not shown). In (B), addition of 0.13 mN LADH sites to 0.2 mM TFE gives the resonance shown, at the same chemical shift. In (C), addition of NAD to 0.2 mM results in the appearance of a new line at 1.17 ppm upfield from TFA, which is due to the ternary E·NAD·TFE complex. One hundred and eighty transients per spectrum were accumulated at 20 °C, in pH 8.7 50 mM sodium pyrophosphate buffer, in part A, 1200 were accumulated in part B, and 2000 were accumulated in part C, in order to help resolve the two peaks observed. In all spectra, the ratio of the area under the TFE peak(s) to the area under the internal standard TFA peak (not shown) is constant. ^{19}F was observed at 141.183 MHz by using an acquisition time of 410 ms and a recycle time of 14.5 s.

VA13A evaluates derivatives of the form $\partial[\sum(\Delta F_{calcd} - \Delta F_{obsd})^2]/\partial(\text{parameter})$ to get new estimates of the parameters ΔF_1, ΔF_2, ψ_1, and ψ_2. New values for parameters are then used to calculate new values for x for each x_0 in the titration, and the above minimization process is repeated until the change in $\sum(\Delta F_{calcd} - \Delta F_{obsd})^2$ is less than a specified number like 0.001. In practice, ΔF_2 as determined above was found to be somewhat dependent on the number of points at the end of the titration used in the plot of $([NAD]_0 - E_0)^{-1}$ vs. ΔF_{obsd}^{-1}. A small range of values of ΔF_2 generally resulted from varying the number of points. The final value of ΔF_2 used was the one resulting in a minimum for the quantity $\sum(\Delta F_{calcd} - \Delta F_{obsd})^2$, when the whole binding isotherm was fitted with ΔF_1, ΔF_2, ψ_1, and ψ_2.

Ligand-Displacement Studies. Experimental work (see Results) suggests that the predominant path of ligand desorption from the ternary complex E·NAD·TFE involves desorption of TFE. Thus, the desorption pathway for displacement of TFE with pyrazole (P) can be written as

$$\text{E·N·TFE} \underset{k_{-1},\ \text{TFE}}{\overset{k_1}{\rightleftharpoons}} \text{E·N} \underset{k_{-2}}{\overset{k_2,\ \text{P}}{\rightleftharpoons}} \text{E·N·P}$$

When a steady-state assumption is used to describe the amount of E·N at any time, the time course of formation of E·N·P from E·N·TFE will appear to be first order with an apparent rate constant, k_{app}, equal to

$$\frac{k_2[\text{P}](k_{-2} + k_1)}{k_{-1}[\text{TFE}] + k_2[\text{P}]} - k_{-2} \qquad (4)$$

When $([TFE]/[P])(k_{-1}/k_2) \ll 1$, which will probably be true when $[TFE] \ll [P]$, k_{app} reduces to k_1, the desorption rate of TFE from E·N·TFE.

For the displacement of pyrazole in E·N·P by TFE, under conditions of saturation of E·N with TFE or pyrazole, $d[E·N·P]/dt = -d[E·N·TFE]/dt$. If $[TFE] \gg [P]$, k_{app} will reduce to the off rate for pyrazole, k_{-2}.

Table I: TFE–LADH Interaction As Measured by the TFE Chemical Shift

complex	pH	net shift from free TFE[b]
E·TFE	8.7, 20 °C	0.00
E·NAD·TFE[a]	6.20	0.22
	6.51	0.28
	6.78	0.28
	7.47	0.25
	8.20	0.27
	8.87	0.23

[a] LADH (0.180 mN), 0.563 mM NAD, and 0.533 mM TFE-*1*-d_2, 1 °C, in 0.01 M pH 6.2 phosphate buffer were titrated with pH 10.5 0.01 or 0.1 M glycine buffer to adjust the pH. Chemical shift was determined from the best-fit Lorentzian line. Spectra consisted of two slow-exchange peaks assigned to free trifluoroethanol (1.395 ppm upfield from TFA, independent of pH from 6 to 9) and the ternary E·NAD·trifluoroethanol complex (see Figure 1C). [b] ppm downfield from free TFE.

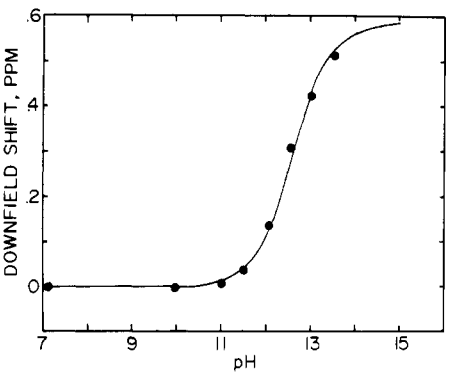


FIGURE 2: TFE-*1*-d_2 ionization as a function of pH as observed by ^{19}F NMR at 20 °C. A solution of 0.3 mM TFE-*1*-d_2 in 0.1 M pH 7.1 sodium phosphate buffer was adjusted up in pH with 1 N NaOH. The ^{19}F chemical shift was initially monitored relative to the internal standard *p*-carboxybenzotrifluoride, which has a pK_a of ca. 3.3, and then referenced to TFE at pH 7.1. The best-fit pK_a was 12.61 with a shift upon ionization of 0.59 ppm.

Results

Figure 1 shows the ^{19}F NMR spectra observed for free TFE and TFE in the presence of LADH. The spectrum in Figure 1A shows the resonance due to free TFE, occurring 1.36 ppm upfield from the internal standard trifluoroacetate (TFA, not shown). The resonance shown here is broadened because the NMR tube is not spun to average out field inhomogeneity broadening. Such spinning results in denaturation of LADH under these conditions. Addition of LADH to excess TFE to form a binary E·TFE complex gives the spectrum shown in Figure 1B. No change in the chemical shift of the peak occurs, although it is somewhat broader. This broadening can be used to quantitate binding (see below). After addition of excess NAD, the spectrum in Figure 1C results. A new peak is formed 1.17 ppm upfield from TFA. It is 40 Hz broader than the TFA resonance and reflects the formation of a ternary complex of LADH, NAD, and TFE. In this spectrum, some free TFE is also present as deduced from relaxation rate measurements. The chemical exchange between the ternary complex and other forms of TFE is slow, since two distinct peaks are present in Figure 1C.

The chemical shift of TFE in the C·NAD·TFE complex is essentially independent of pH over the range from pH 6.20 to 8.9 as shown in Table I. The shift of free TFE upon ionization is shown in Figure 2. In this case, the chemical

shift of TFE is clearly dependent on the pH of the solution, with a downfield shift occurring above pH 11. Since in this region the alcohol is beginning to ionize (Takahashi et al., 1971), the ionization is being reflected in a fast-exchange average line between neutral and ionized alcohol. These data can be treated as a single ionization curve, according to

$$\delta_{obsd} = \delta_{BH} + \left(\frac{K_a}{K_a + [H]}\right)\delta_{B^-} \quad (5)$$

where δ_{obsd}, δ_{BH}, and δ_{B^-} are the observed fast-exchange averaged shift, the shift of neutral alcohol, and the shift of fully ionized alcohol, respectively, and K_a is the association constant for the proton. This treatment yields a pK_a of 12.6 and a shift for $CF_3CD_2O^-$ of 0.59 ppm downfield from the shift of the neutral alcohol. The solid curve calculated by using these parameters is shown overlaying the data in Figure 2.

To help further interpret the bound shift of TFE in the E·NAD·TFE complex, we generated two covalent adducts of zinc and the alcohol. Diphenylzinc or diethylzinc was reacted with dry TFE in C_6D_6 to form the covalent zincate. The TFE resonance was shifted 3.4 ppm upfield in the C_6H_5–Zn–OCH_2CF_3 complex and 1.7 ppm in the Et–Zn–OCH_2CF_3 complex.

TFE Equilibrium Binding in Binary Complexes. In various mixtures of LADH and TFE, only a single-exponential T_2 relaxation process is observed for the trifluoromethyl resonance. The value of the apparent relaxation time varies with the ratio of TFE to LADH. This suggests that the exchange of TFE between its bound and free environments is comparable or faster than the bound and the free T_2 relaxation rates. By observing how this average value varies as a function of ligand concentration at constant enzyme concentration, according to

$$L_0 = E_0\frac{(1/T_{2M} - 1/T_{2f})}{(1/T_{2,app} - 1/T_{2f})} - K_d \quad (6)$$

we can determine the binding constant of TFE to the enzyme, K_d, and the apparent bound T_2 relaxation time, T_{2M}. Here $1/T_{2,app}$ is the measured, average relaxation rate, $1/T_{2f}$ is the relaxation rate of the ligand in the absence of binding, and E_0 and L_0 are the concentrations of total enzyme and free ligand, respectively. Here the interpretation of T_{2M} is $1/T_{2M} = 1/(T_{2b} + \tau)$ where T_{2b} is the intrinsic relaxation rate of the bound TFE and τ is its bound lifetime. For our conditions, the free ligand is nearly equal to the total concentration of ligand. Thus, a plot of $(1/T_{2,app} - 1/T_{2f})^{-1}$ vs. L_0 should yield a straight line of slope equal to $E_0(1/T_{2M} - 1/T_{2f})$ and intercept equal to $-K_d$. Such a plot for TFE and LADH is shown in Figure 3 at pH 8.75, 20 °C. The values of K_d and T_{2M} are 1.4 mM and 9.8 ms, respectively. The linear fit of the data shown in Figure 3 suggests that a single, noninteracting binding constant can be used to describe TFE binding to both LADH subunits. The data in Figure 3 were also fit to the binding equation without the assumption that L_0 and free L are equal by using a nonlinear least-squares technique. No changes in the value of K_d and T_{2M} were found.

NAD Equilibrium Binding in Ternary Complexes. The binding of NAD to enzyme saturated with TFE and to enzyme saturated with pyrazole, another substrate analogue, was monitored by the quench of LADH fluorescence upon NAD binding. These experiments were designed to examine the possibility of cooperative interactions in ternary complexes.

For enzyme saturation with either substrate analogue, fluorescence quench is a linear function of NAD binding, as shown in Figure 4. Figure 4A shows a titration of 72 μN LADH with NAD at pH 7.5 in the presence of 12.8 mM

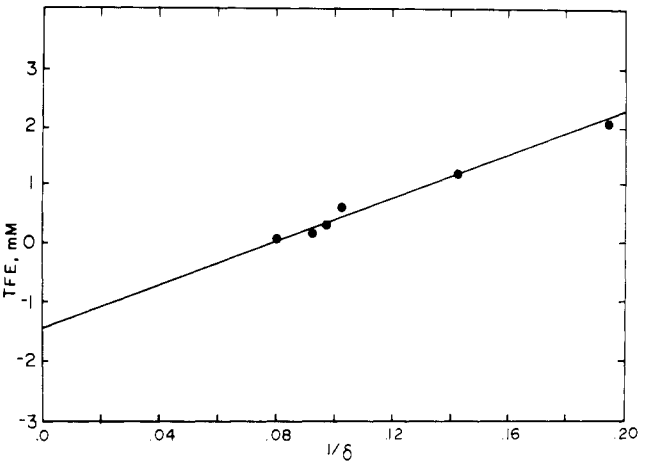


FIGURE 3: Trifluoroethanol-*1*-d_2 binding to LADH at pH 8.85, 20 °C, determined by spin–spin relaxation requirements. δ represents $1/T_{2,app} - 1/T_{2f}$; LADH was 0.19 mN; TFE concentration was varied from 0.08 to 2.3 mM. See Materials and Methods for treatment of the data. The best-fit K_d was 1.4 mM.

pyrazole, monitored by the formation of the E·NAD·pyrazole adduct on the enzyme surface. This was monitored by its absorption at 300 nm (Theorell & Yonetani, 1963). The same titration is shown by fluorescence quench of the enzyme in Figure 4B. Part C shows an identical fluorescence titration of LADH with NAD in the presence of 12.8 mM TFE. The titration by absorption establishes tight binding of NAD to enzyme in the presence of pyrazole, and establishes the shape of the binding curve (Figure 4A). Figure 4B is virtually identical with 4A. Thus, NAD binding in the presence of pyrazole or TFE displays a linear dependence of the fluorescence signal change on NAD binding.

Representative binding isotherms are shown in Figure 5A for NAD binding in the presence of saturating (3.4 mM) TFE at pH 8.75 and in Figure 5B for NAD binding in the presence of saturating (15.9 mM) pyrazole at pH 7.5. The best-fit curves are shown overlaying the data in each plot. In the TFE case, the best-fit parameters were $\psi_1 = 49\ \mu M^{-1}$ and $\psi_2 = 1040\ \mu M^{-2}$ with an overall fluorescence quench of 47%. Similarly, for pyrazole the best-fit parameters were $\psi_1 = 189\ \mu M^{-1}$ and $\psi_2 = 7360\ \mu M^{-2}$ with an overall fluorescence quench of 38%.

The extent of subunit interactions can be estimated by examining the ratio of the dissociation constants for the binding of the first and second ligands, K_1/K_2, which is equal to $[4\psi_2/(\psi_1)^2]^{1/2}$ for a dimeric protein of identical sites. This gives values of $K_1/K_2 = 1.7$ and 0.82 for TFE- and pyrazole-saturated LADH, respectively. These values correspond to Hill coefficient values of 1.13 for NAD binding in the presence of TFE and 0.95 in the presence of pyrazole. For five separate experiments, an average Hill coefficient of 1.16, with a range from 1.04 to 1.26, was obtained in the presence of TFE. For an equal number of experiments, an average Hill coefficient of 0.89, with a range of 0.72–0.98, was obtained in the presence of pyrazole. These values are within our experimental error of no observed cooperativity.

For determination of the concentration dependence of TFE and pyrazole for ternary complex formation with E·NAD, NAD binding isotherms were determined as a function of the concentration of these ligands. Binding of NAD to E·pyrazole was measured at pH 7.5. At higher pH values, its binding is too tight to measure by our techniques. Binding of NAD to the E·TFE complex, which is about 30-fold weaker, was measured at pH 8.75. Total TFE or pyrazole concentrations were kept at least 10-fold above enzyme site concentration so that a small proportion of these ligands were bound under all

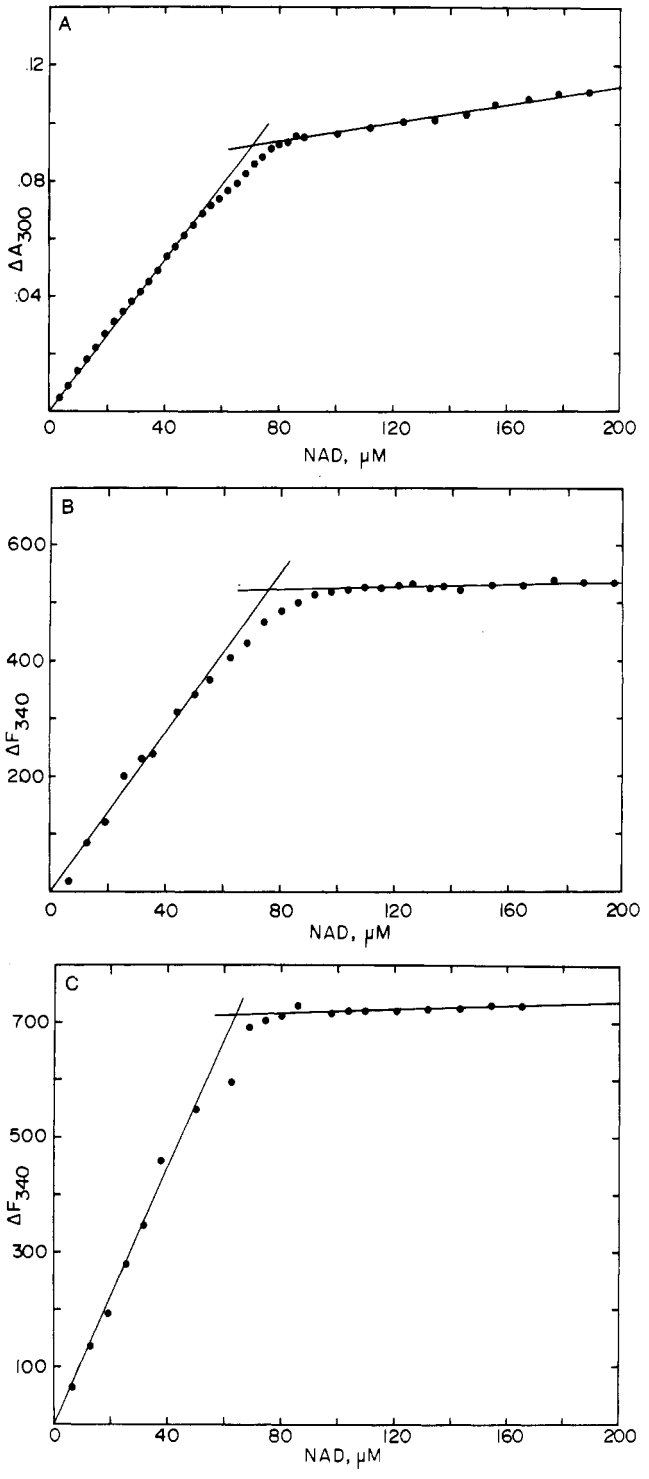


FIGURE 4: Linearity of quench of LADH fluorescence upon binding of NAD in the presence of pyrazole or TFE. (A) NAD binding by absorbance measurement of E·NAD·pyrazole formation at 300 nm. [LADH] = 72 μN and [pyrazole] = 13 mM at pH 7.5 in 0.1 M sodium phosphate buffer at 25 °C. (B) NAD binding to 72 μN LADH + 13 mM pyrazole in 0.1 M sodium phosphate buffer at 25 °C. The quench of fluorescence is now observed. $\lambda_{excitation}$ = 300 nm, $\lambda_{emission}$ = 340 nm. (C) Binding of NAD to 72 μN LADH + 13 mM TFE in pH 8.7 50 mM sodium pyrophosphate buffer at 25 °C. Excitation and emission as in part B.

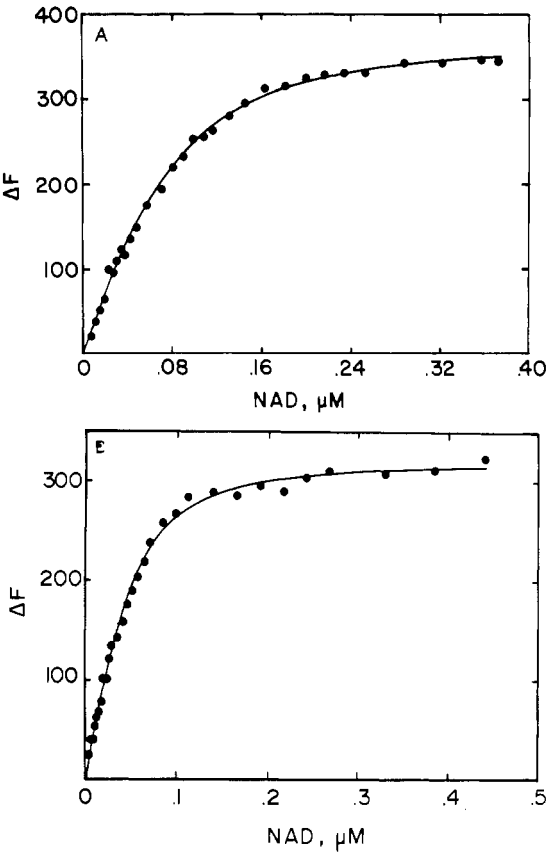


FIGURE 5: (A) Isotherms for NAD binding to 0.064 μN LADH + 3.4 mM TFE at pH 8.7, 25 °C, in 50 mM sodium pyrophosphate buffer. ΔF on the y axis increases with fluorescence *quench*, in arbitrary units. The best-fit curve derived from the nonlinear least-squares approach described in the text is overlaid on the data. The derived best-fit values were $\Delta F_1 = 189$, $\Delta F_2 = 377$, $\psi_1 = 49\ \mu M^{-1}$, and $\psi_2 = 1040\ \mu M^{-2}$. Since fluorescence quench was linear with binding (cf. Figure 4), ΔF_1 was fixed at $0.5(\Delta F_2)$ (see text). (B) Isotherm for NAD binding to 0.056 μN LADH + 15.9 mM pyrazole in pH 8.7 50 mM sodium pyrophosphate buffer at 25 °C. The best-fit curve is overlaid on the data and is described by $\Delta F_1 = 163$, $\Delta F_2 = 326$, $\psi_1 = 189\ \mu M^{-1}$, and $\psi_2 = 7360\ \mu M^{-2}$.

conditions. Figure 6 shows the measured affinity of NAD for LADH as a function of varying amounts of TFE (Figure 6A) and pyrazole (Figure 6B). The logarithm of the half-saturating NAD concentrations, $N_{0.5}$, is plotted against the logarithm of the concentration of free ligand, where the x-axis ligand is TFE or pyrazole.

This type of plot can be used to extract considerable quantitative information about the interaction of both ligand pairs (NAD·TFE and NAD·pyrazole) with the enzyme [see Dixon (1953) for an application using H^+ as a second ligand]. Thus, one can estimate the value of the dissociation constant of TFE from the free enzyme as 1.3 mM while it is substantially tightened in the presence of saturating NAD to a value of 6 μM, a 5000-fold enhancement. The solid curves shown in Figure 6 are calculated by using these values and the dissociation constants for NAD given in Table II.

The effect of TFE on NAD binding to the enzyme appears to saturate at about 1 mM, while at pH 7.5 the binding of pyrazole is saturated somewhat above 10 mM. NAD binding is tightened by a factor of about 200-fold at TFE saturation, while for pyrazole it appears to be tightened by about 4600-fold. At saturation with TFE or pyrazole, NAD binding is very tight, with a K_d of 33 nM from E·NAD·TFE and 10 nM from E·NAD·pyrazole under the conditions of the binding experiments.

Kinetics of Ligand Binding. The phenomenological desorption rates of TFE from E·NAD·TFE were measured by NMR relaxation techniques at pH values where the desorption rate was too fast to conveniently measure by using a standard mixing technique. Under these conditions, where the exchange of TFE on and off the ternary complex is slow relative to the

Table II: Dissociation Constants for NAD, Pyrazole, and TFE from Binary and Ternary Complexes with LADH

variable	value	calcd from	independent values	K_0 measured
		TFE, pH 8.75		
K_T	1.3 mM	high TFE axis intercept of Figure 6A	1.4 mM[a]	TFE from E·TFE
K_T'	6.0 μM	low TFE axis intercept		TFE from E·NAD·TFE
K_N	6.2 μM	low TFE y-axis asymptote	12–40 μM[b]	NAD from E·NAD
K_N'	33 nM	high TFE y-axis asymptote		NAD from E·NAD·TFE
		Pyrazole, pH 7.5		
K_p	9.1 mM	high pyrazole x-axis intercept, Figure 6B	14 mM[c] 0.16 mM[d]	pyrazole from E·pyrazole
K_p'	1.7 μM	low pyrazole x-axis intercept		pyrazole from E·NAD·pyrazole
K_N''	46 μM	low pyrazole y-axis asymptote	ca. 80 μM[e]	NAD from E·NAD
K_N'''	10 nM	high pyrazole y-axis asymptote		NAD from E·NAD·pyrazole

[a] As determined by spin-spin relaxation measurements (see Results); 590 mM, pH 7.0 (Sigman, 1967). [b] Literature values range from 12 to 40 μM at pH 8.75 (Sund & Theorell, 1963; Taniguchi, 1967). The asymptotic value of K_N was determined when [TFE] = 0. [c] Sigman (1967), pH 7.0. [d] Theorell & Yonetani (1963), pH 7.0. [e] From Sund & Theorell (1963) and Taniguchi (1967). The asymptotic value of K_N'' was determined when [pyrazole] = 0.

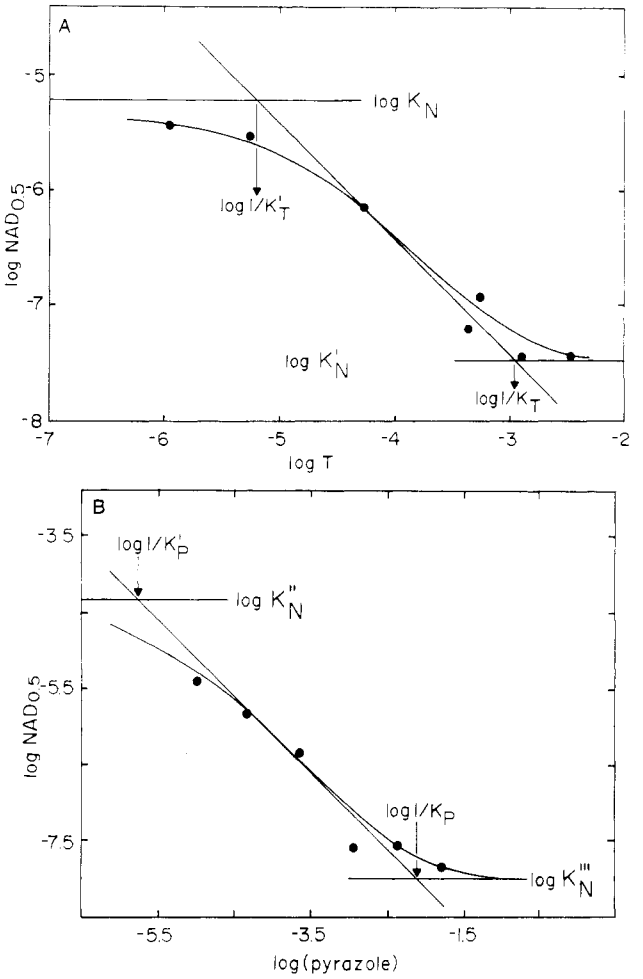
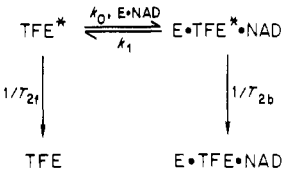


FIGURE 6: (A) NAD binding to LADH + TFE in pH 8.7, 25 °C, 50 mM sodium pyrophosphate buffer as the concentration of TFE is varied. LADH concentration was 0.06–0.14 μN; NAD concentration was varied from 0 to 44 μM when no TFE was present, or from 0 to 0.4 mM when 3.4 mM TFE was present. For parts A and B, LADH was excited at 300 nm, and fluorescence was observed at 340 nm. See text for details of data analysis. (B) NAD binding to LADH + pyrazole as a function of pyrazole concentration in pH 7.5 0.1 M sodium phosphate buffer at 25 °C. LADH concentration was 0.07–0.14 μN in sites; NAD concentration varied from 0 to 100 μM when [pyrazole] = 0, or from 0 to 0.5 μN when [pyrazole] = 15.9 mM. See Materials and Methods for other details of the experiment and also Table II for results.

chemical shift difference between the free and bound environments of about 170 s^{-1}, the chemical exchange process acts as a relaxation process. This can be seen by considering the following kinetic scheme which describes the relaxation of TFE in the free and bound environments:



Here TFE* and E·TFE·NAD* represent the excess spin populations in the free and bound environments, respectively. These can interconvert by the chemical processes of combination with E·NAD with a pseudo-first-order rate of k_0[E·NAD] or by desorption at a rate k_1. Relaxation can occur at rates $1/T_{2f}$ and $1/T_{2b}$ in the free and bound environments. If the rate of desorption, k_1, is much slower than the bound rate of relaxation, $1/T_{2b}$, then every time a TFE* is bound it will relax. Thus, the rate of binding adds to the rate of relaxation in the bound state. We can write a quantitative expression for the rate of relaxation in the free state ($1/T_{2,app}$) as

$$1/T_{2,app} = \frac{1}{T_{2f}} + k_0[\text{E·NAD}] \tag{7}$$

where [E·NAD] is the chemical concentration of E·NAD complexes. If K_T is the dissociation constant for TFE from E·NAD·TFE and E_0 represents the total concentration of enzyme saturated with NAD, then

$$\frac{1}{T_{2,app}} = \frac{1}{T_{2f}} + k_0 E_0 \frac{K_T}{K_T + [\text{TFE}]} \tag{8}$$

We know $K_T \ll$ [TFE] under our conditions, so that

$$\frac{1}{T_{2,app}} = \frac{1}{T_{2f}} + k_0 E_0 \frac{k_T}{[\text{TFE}]} = \frac{1}{T_{2f}} + \frac{k_1 E_0}{[\text{TFE}]_0 - E_0} \tag{9}$$

where $[\text{TFE}]_0$ is the total concentration of trifluoroethanol and $[\text{TFE}]_0 - E_0$ is the free concentration of trifluoroethanol. Defining T_{obsd} as

$$T_{obsd} = \left(\frac{1}{T_{2,app}} - \frac{1}{T_f} \right)^{-1} = \frac{[\text{TFE}]_0 - E_0}{k_1 E_0} \tag{10}$$

a plot of T_{obsd} vs. $[\text{TFE}]_0 - E_0$ gives a slope of $1/(k_1 E_0)$ and an x-axis intercept of zero. Figure 7 gives the results of this procedure at pH* 6.05, 6.40, and 6.75; 6.75 was an upper limit in pH for these experiments since at higher pH values $1/T_{2,app} = 1/T_{2,free}$ and no additional line width can be used to determine off rates. Linear regression analysis gave observed

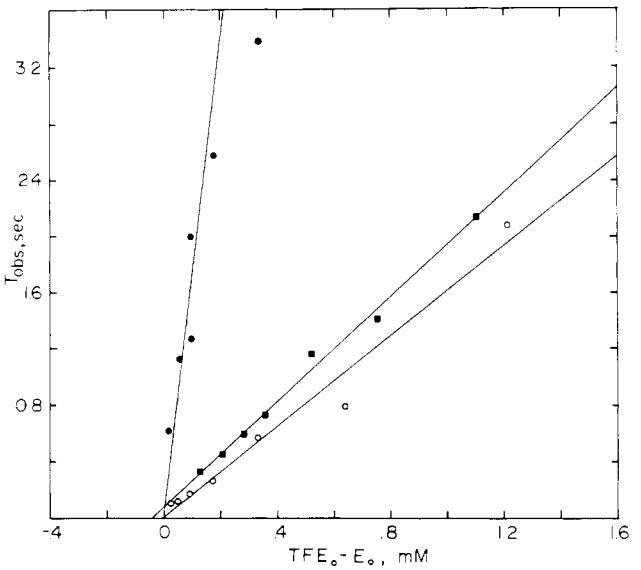


FIGURE 7: Observed lifetime of TFE as a function of the concentration of free TFE as determined by ^{19}F NMR spin–spin relaxation measurements. The derived desorption rates of TFE from E·NAD·TFE at pH values of 6.75 (●), 6.40 (○), and 6.05 (■) are 0.6, 2.7, and 1.3 s^{-1} (see Materials and Methods). In all cases, 0.1 M sodium phosphate buffer was adjusted to the pH shown on the figure. At pH 6.75, E_0 = 0.19 mN and $[NAD]_0$ = 0.38 mM; at pH 6.40, E_0 = 0.20 mN and $[NAD]_0$ = 0.40 mM; at pH 6.05, E_0 = 0.23 mM and $[NAD]_0$ = 0.47 mM. T_{2f} was determined by the addition of pyrazole to 0.1 M.

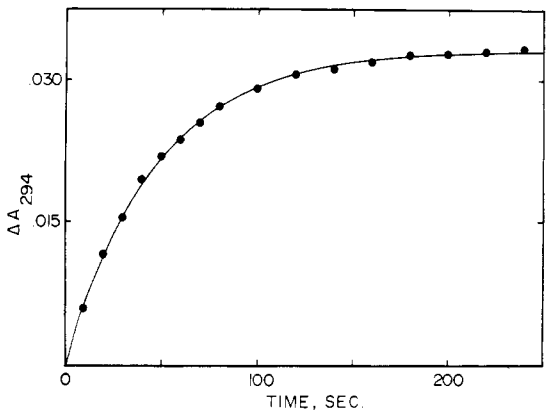


FIGURE 8: Time course of displacement of TFE from E·NAD·TFE with pyrazole. LADH (10 μN) was preincubated with 2 mM NAD and 16 mM TFE in pH 8.7 50 mM sodium pyrophosphate buffer at 25 °C. TFE was displaced with 40 mM pyrazole. The best-fit rate constant to a single exponential was 0.025 s^{-1}.

off rates, $1/T_{obsd}$, of 1.3, 2.7, and 0.6 s^{-1} at pH 6.05, 6.40, and 6.75, respectively. The increase in relaxation rate at higher temperatures is consistent with the view that slow exchange conditions are satisfied here.

TFE desorption from E·NAD·TFE at pH values above 6.75 was investigated by direct displacement with pyrazole. The differential absorption of E·NAD·TFE and E·NAD·pyrazole at 294 nm allows the formation of the latter complex to be monitored with little interference from nucleotide or protein absorption. Slower apparent off rates at higher pH are accessible. Figure 8 shows the formation of the E·NAD·pyrazole complex at pH 8.8, 20 °C. The curve showed no deviation from a first-order reaction, giving a first-order rate constant of 0.025 s^{-1}.

Phenomenological rate constants for displacement of TFE monitored by formation of E·NAD·pyrazole were obtained in the above fashion from pH 8.0 to 10.0. The curves were well described as first-order processes in this pH range. A plot of

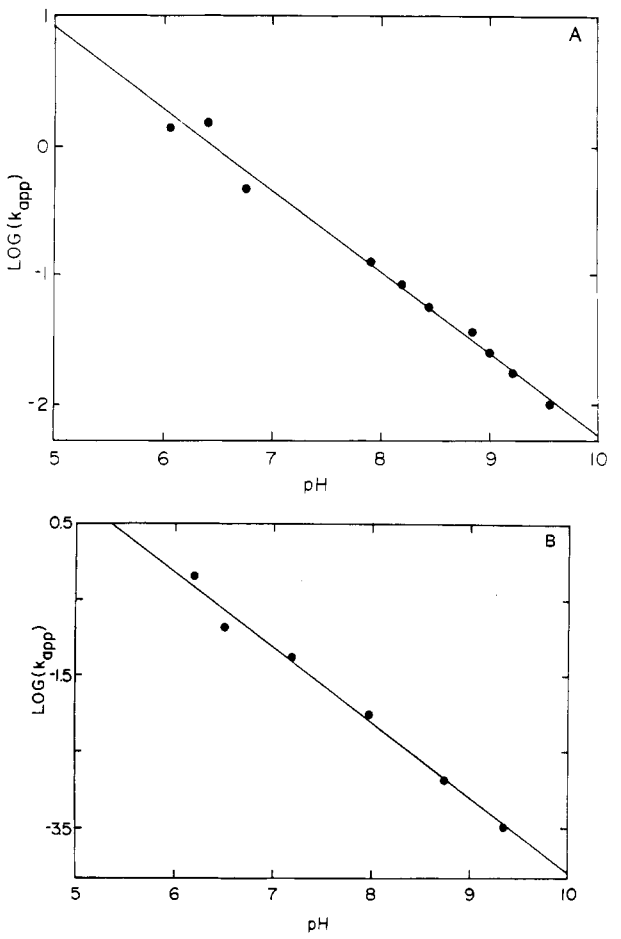


FIGURE 9: (A) pH dependence of the apparent off rate constant of TFE from E·NAD·TFE by pyrazole displacement. The three points at lowest pH are based on TFE self-exchange measurements presented in Figure 7. All other points between pH 7.9 and 9.6 are by direct displacement of TFE with pyrazole. LADH at 10 μN, 2 mM NAD, and 16 mM TFE were preincubated. Sodium pyrophosphate buffer (50 μM) was used, at 25 °C. The slope of the best-fit line is −0.80 ± 0.01. pH measurements were made after displacement was complete. (B) pH dependence of the apparent off rate constant of pyrazole from E·NAD·pyrazole when displaced by TFE. Sodium pyrophosphate buffer (50 mM) was used for pH values above 8; 0.1 M sodium phosphate buffer was used for values below pH 8, all at 25 °C. LADH (10 μN), 2 mM NAD, and 2 mM pyrazole were preincubated. Pyrazole was displaced from the ternary complex with TFE, giving a final concentration of 230 mM. pH was measured after the displacement was complete.

log k_{obsd} vs. pH is presented in Figure 9A, including results from NMR measurements. Between pH 7 and 10, there are no break points in the plot. There may be a plateau in the observed value of k_{app} below pH 6.5, suggesting the possible involvement of a group of this pK_a in the desorption kinetics. Linear regression analysis gives a best-fit slope of −0.8 ± 0.1 between pH 7 and 10. Note that data points from the NMR measurements lie on the same line as measurements made at higher pH by using the direct displacement method.

In order to obtain more microscopic information about the mechanism of this displacement reaction, we monitored the NAD dependence of the observed rate of TFE displacement by pyrazole. LADH (18 μN) was preincubated with 175 mM TFE and differing concentrations of NAD at pH 8.75, 25 °C. TFE was then displaced with 50 mM pyrazole. Little difference in the observed rate was noticed: with 1 mM NAD, k_{obsd} = 0.015 s^{-1}, while preincubating with 45 mM NAD gave a k_{obsd} of 0.012 s^{-1}. Thus, the observed rate seems essentially independent of NAD concentration.

Pyrazole was displaced from E·NAD·pyrazole with high concentrations of TFE, as a function of pH. All kinetic traces were well described as first order. Figure 9B shows the pH dependence of the log of the rate of disappearance of E·NAD·pyrazole from pH 6 to 10. The data have a best-fit line with a slope of -1.01 ± 0.01. As in the case of TFE displacement with pyrazole, the rates drop linearly with pH, with no break points or curvature in the plot.

Discussion

^{19}F NMR offers a sensitive technique to probe the environment of the TFE in its ternary complex with NAD. We are particularly interested in the ionization properties of the bound TFE because the equilibrium studies of Laws & Shore (1978) have demonstrated that a proton is released upon formation of the ternary complex of LADH, NAD, and TFE. This is particularly important since it has been suggested by Kvassman & Pettersson (1980a,b) that TFE is bound as the anion, implying that TFE is at least partly responsible for the release of the proton. We find no evidence for a chemical shift of the TFE resonance upon binding to LADH in the absence of NAD, or in the presence of NADH (data not shown). Upon formation of the ternary complex with NAD, the TFE resonance shifts slightly downfield by about 0.28 ppm from its free position. This shift is independent of pH over the range from 6.2 to 8.9. However, from the model studies reported here, shifts on the order of 1 ppm would be expected if a change in the ionization state of TFE occurs over this pH range. Thus, it is unlikely that the bound TFE has a pK_a in the range 6.2–8.9.

The remaining problem is to establish the state of ionization of the bound TFE in this pH range. A number of possibilities must be considered. These include (1) the formation of an unneutralized TFE anion in the bound state, (2) the formation of a covalent adduct with NAD, (3) the formation of a zinc alkoxide bound with the active-site zinc ion, and (4) the formation of a neutral alcohol with the proton release involving a protein residue whose pK_a is dramatically shifted as a result of ternary complex formation.

The first alternative of a bound TFE anion seems an unlikely explanation. The observed shift upon ternary complex formation is only about half the magnitude observed for TFE ionization in solution. Further, the data of Laws & Shore (1978) suggest that the formation of the ternary complex perturbs a group of $pK_a = 8.6$ to a much lower value to account for the proton release. Formally this pK_a could be due to an ionizable group on either the enzyme, NAD, or TFE in their free states. We measure the pK_a of free TFE to be about 12.6. It is unlikely that its pK_a would be lowered by at least 6 units to fall below 6.2.

If the TFE were to form a covalent adduct with NAD when bound on the enzyme surface, as has been suggested for pyrazole (Theorell & Yonetani, 1963), this would result in the release of a proton and would most likely also result in the perturbation of the bound coenzyme electronic spectrum. An additional absorption band appears in the E·NAD·pyrazole complex, around 294 nm, but we have not observed any new absorption bands in the region from 270 to 400 nm in the E·NAD·TFE complex. This argues against the formation of an adduct with an NADH-like structure which could provide this new absorption.

We have also examined the ^{19}F chemical shift of TFE in a nonpolar solvent after replacement of the alcoholic hydrogen with zinc by reaction of TFE with diethylzinc or diphenylzinc in anhydrous C_6D_6. These complexes produce upfield shifts of 1.7 and 3.4 ppm, respectively, and are meant to provide information as to the shift expected upon formation of a covalent zinc–alkoxide bond. However, the LADH active-site zinc is liganded by two cysteines and a histidine from the protein, and these model compounds do not provide these other ligands. It is likely, however, that the chemical shift of TFE will be most sensitive to the nature of the alkoxide bond and would probably be only secondarily affected by the other zinc ligands.

Thus, the formation of the trifluoromethyl ethoxide ion in aqueous solution or its covalent zinc adduct in C_6D_6 leads to large chemical shifts of its ^{19}F resonance. Binary complex TFE and TFE in the E·NADH·TFE abortive ternary complex both exhibit no observable ^{19}F chemical shift, while the peak due to TFE in its ternary complex with NAD and the enzyme shifts only 0.28 ppm downfield. From our data, the most reasonable possible ionization states of TFE in its ternary complex with LADH and NAD are that it is bound as the neutral alcohol or bears a partial negative charge. More experiments are necessary to unambiguously determine the ionization state of the bound TFE. However, we are very confident that it does not have a pK_a between 6.2 and 8.9.

Our NMR observations allow us to gain some insight into the mechanism. The linear increase in the apparent TFE off rate with proton concentration in the pH range 7–10 (Figure 9A) implies that TFE desorbs more rapidly when a certain residue in the complex is in its protonated form. This suggests TFE might thus bind more tightly to the ionized form of a group at the active site [see also Shore et al. (1974)]. Recently, Kvassman & Pettersson (1980a,b) have suggested a pK_a of 6.4 for the ionizable group involved in TFE desorption. Our data at low pH are consistent with this view. It is interesting that the spectrum of bound TFE shows no shift at pH values as low as 6.2. This suggests that the bound TFE is not the group with the pK_a of 6.4. If the released proton is not coming from TFE, it may instead be provided by a residue at the active site. There appear to be several possible proton donors at the active site which could provide the released proton upon TFE binding. Of the two cysteines that are present, Cys-46 is selectively alkylated by iodoacetate and Cys-174 is not modified. Thus, Cys-46 may be available as a proton donor. It is possible that cysteine ionization could account for the pK_a of 9.6 in the free enzyme, which appears to be perturbed to 7.6 upon NAD binding, and to below 4.5 in the E·NAD·TFE ternary complex. A single water molecule at the active site has also been discussed as a possibility (Sloan et al., 1975; Dworschack & Plapp, 1977; Maret et al., 1979; Drysdale & Hollis, 1980), while Cook & Cleland (1981b) have suggested that the state of ionization of His-51 controls alcohol binding at the active site with a pK_a value near 7. It is clear that an ionizable group of $pK_a = 7$ has a negligible effect on the ionization state of bound TFE and therefore little effect on the affinity for TFE in the ternary complex. Although evidence from modified enzyme studies appears to rule out all zinc ligands, it was suggested that ionizations affecting the quench of fluorescence are not necessarily relevant to active-site catalytic proton donors (Parker et al., 1978). Thus, the identity of the active-site proton donor is still uncertain, although our studies are most consistent with a role for a protein side chain. It is also possible that TFE bound in ternary complexes assumes a partial negative charge, with the remaining portion of that charge distributed elsewhere, perhaps in the active-site ligands.

Using the trifluoromethyl moiety of this inactive alcohol as a probe, we have been able to examine the equilibrium rate of exchange of TFE between its ternary complex and free TFE

by using ^{19}F transverse relaxation time measurements. These self-exchange experiments, which should be limited by the desorption rate of TFE from the ternary complex, can be done in the pH range of 6.0–6.7. Above this range, the information can be supplemented by results from complementary ligand-displacement experiments, which rely on the direct displacement of TFE with pyrazole, another ethanol-competitive inhibitor which undergoes spectral changes in its ternary complex with LADH and NAD. Thus, the pH dependence of the TFE desorption rate can be studied between pH 6 and 9.6.

Since the enzyme is a dimer, it is possible in the displacement studies that the presence of a NAD·pyrazole ternary complex at one active site could affect the rate of TFE desorption from the other subunit. However, the rates of self-exchange as seen by NMR lie on the same line as rates from pyrazole displacement measured at different pH values. This suggests that there are no large kinetic effects which result from placement of TFE with pyrazole on the enzyme surface as opposed to TFE replacement by another TFE molecule. The observed displacement is essentially independent of the concentration of NAD preincubated with the enzyme and TFE even at 45 mM NAD. This suggests that the observed behavior is due to TFE desorbing from LADH·NAD, the same sequence of events thought to occur with aromatic alcohol substrates [see Wratten & Cleland (1965)] and cyclohexanol (Cook & Cleland, 1981a).

The time course of TFE desorption as monitored by pyrazole displacement shown in Figure 8 is well described as a single exponential. This implies that the two bound TFE molecules desorb at the same rate. This suggests that there is no site–site interaction in TFE binding to NAD-saturated enzyme. Similar observations and conclusions apply to the desorption kinetics of pyrazole to the NAD-saturated enzyme.

The slow desorption rate of TFE at pH 8.75 of 0.025 s^{-1} suggests its use as a kinetic inhibitor analogous to pyrazole [see McFarland & Bernhard (1972)]. Since its desorption rate is 50-fold or more slower than the turnover rate of LADH, TFE can be used to trap E·NAD complexes to prevent their further turnover in stopped-flow aldehyde reduction experiments. Since formation of E·NAD·TFE results in no formation of new absorption bands in the 270–400-nm region, unlike the case for pyrazole, no corrections for the time course of absorption changes due to this complex will be necessary.

Using the intrinsic fluorescence of the protein tryptophan residues, we can demonstrate a linear dependence of the fluorescence quench on NAD binding in the presence of TFE or pyrazole (Figure 4A–C) by comparison with binding monitored by absorption measurements. Thus, the quench of the protein fluorescence can be used to monitor the shape of the NAD binding isotherm in the presence of TFE or pyrazole, an experiment which inquires about any thermodynamic interactions between the two protein active sites when NAD binds to form these ternary complexes. NAD binding isotherms in the presence of 3.4 mM TFE (Figure 5A) or 15.9 mM pyrazole (Figure 5B) reveal little evidence for cooperative effects in NAD binding in ternary complexes.[1] In accord with previous data, NAD binding isotherms to LADH in the absence of TFE or pyrazole are also hyperbolic (Sund & Theorell, 1963).

We can also monitor the binding isotherms of NAD at a variety of TFE and pyrazole concentrations (Figure 6A,B). There is a strong synergistic interaction between NAD and TFE or pyrazole, such that NAD binding is tightened by a factor of 4600 in the presence of pyrazole at pH 7.5. We can also use these data to extract estimates of the equilibrium dissociation constants for LADH in the absence and presence of saturating NAD, with values for TFE of 1.3 mM and 6 μM, respectively, and values for pyrazole of 9.1 mM and 1.7 μM. The value for TFE in the absence of NAD of 1.3 mM agrees well with our direct measurement of 1.4 mM obtained by ^{19}F transverse relaxation measurements in Figure 3. Thus, it is likely that the NMR site we observe in the binary complex of LADH and TFE is the site which couples to NAD binding and is not a minority species.

Drysdale & Hollis (1980) examined the interactions of TFE with LADH in which the active-site zinc atoms were replaced by cobalt. Their data suggested a distance between the TFE and metal ion which was too great for there to be direct complexation of the TFE to the metal ion itself. For technical reasons, however, they were unable to observe the ternary complex of TFE with NAD and the enzyme.

All of these data taken together virtually rule out any possibility of site–site interactions which could generate cooperative, or anticooperative, binding of NAD or the substrate analogue trifluoroethanol either in binary or in ternary complexes with horse liver alcohol dehydrogenase. In addition, the bound lifetimes for trifluoroethanol and NAD in their ternary complex with LADH are long relative to typical substrate turnover times. In the following paper, we employ these properties of the trifluoroethanol–NAD ligand pair to further examine the question of half of the site's reactivity in this protein.

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[1] In view of the scatter in the data, average Hill coefficients of 0.89 for NAD binding in the presence of pyrazole, or 1.16 for NAD binding in the presence of TFE, reflect essentially hyperbolic binding behavior.

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Biphasic Transient Kinetics Are a Property of a Single Site in Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The transient kinetics under single turnover conditions of the dimeric enzyme horse liver alcohol dehydrogenase (LADH) are often biphasic with nearly equal amplitudes in the fast and slow components of the reaction. These observations have been the basis for the suggestion that this enzyme displays half of the sites reactivity or kinetic site–site interactions in its catalytic cycle [Bernhard, S. A., Dunn, M. F., Luisi, P. L., & Shack, P. (1970) *Biochemistry 9*, 185]. Alternatively, the biphasic kinetics could be the result of a complex mechanistic path at a single site with no kinetic interactions between the active sites [Kvassman, J., & Pettersson, G. (1976) *Eur. J. Biochem. 62*, 279; Kvassman, J., & Pettersson, G. (1978) *Eur. J. Biochem. 87*, 417]. To resolve this question, we have prepared LADH in which one of the two active sites is occupied by the strongly bound, slowly exchanging ternary complex inhibitors NAD with trifluoroethanol or NAD with pyrazole. These half-inhibited dimers show essentially the same biphasic transient kinetics as the native enzyme. These results strongly support mechanisms in which the observed kinetics are a property of each independently functioning site of the dimer. The possibility of half of the sites reactivity or kinetic site–site interaction appears to be ruled out.

The dimeric alcohol dehydrogenase from horse liver (LADH) is one of the simplest oligomeric enzymes thought to possess half-site reactivity (Bernhard et al., 1970; Luisi & Favilla, 1972; Luisi & Bignetti, 1974; Baici & Luisi, 1977). It is one of several dimers thought to be half-sited, including porcine heart malate dehydrogenase (Harada & Wolfe, 1968) and *Escherichia coli* alkaline phosphatase (Lazdunski, 1973). Half-site reactivity in alkaline phosphatase has been strongly challenged (Bloch & Schlesinger, 1973) as has half-site reactivity in a variety of membrane-associated transport proteins (Kyte, 1981). Challenges to the existence of a half-site reactivity in LADH have also been reported (Kvassman & Pettersson, 1976, 1978; Weidig et al., 1977; Kordal & Parsons, 1979; Tatemoto, 1975a,b).

Although a myriad of other enzymes have been reported to display half-sited behavior (Lazdunski, 1973; Levitzki & Koshland, 1974; Seydoux et al., 1974), it is of interest to carefully examine this phenomenon for the simple (in terms of quaternary structure) enzymes. In view of the long controversy involving LADH and its reported kinetic half-sited behavior, we have decided to determine the transient kinetic properties of individual half-inhibited dimers of LADH in solution. Such transient kinetic properties of the native enzyme are the main evidence for the initial suggestion of half-sitedness (Bernhard et al., 1970) or subunit interactions in this system (McFarland & Bernhard, 1972; Dunn et al., 1979).

Our approach to exploring the interpretation of these transient kinetic properties is to obtain an enzyme preparation in which one active site has been blocked and one is normal. We have employed two tightly bound, but not covalently attached, specific inhibitors of LADH to inhibit a given site. These are NAD·pyrazole and NAD·trifluoroethanol (TFE). We are then able to determine the kinetic properties of this enzyme for comparison with the uninhibited enzyme. The distribution of uninhibited (E_2), half-inhibited (E_2I) (where I = inhibitor), and fully inhibited (E_2I_2) dimers can be uniquely determined by using the equilibrium binding isotherms of the inhibitors. We have measured the binding curves for the above

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