Determination of the Equilibrium Distribution between Alcohol and Aldehyde Substrates When Bound to Horse Liver Alcohol Dehydrogenase Using Magnetic Resonance†

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ABSTRACT: The interactions of horse liver alcohol dehydrogenase (LADH) with the *p*-trifluoromethyl derivatives of benzyl alcohol, benzaldehyde, and benzoic acid have been investigated by use of ^{19}F nuclear magnetic resonance. The aldehyde and alcohol are good substrates for the enzyme and display kinetic characteristics similar to other alcohol/aldehyde pairs. In single-turnover experiments with NADH, *p*-(trifluoromethyl)benzaldehyde shows biphasic kinetics similar to those shown by other aromatic aldehydes, which led Bernhard and co-workers [Bernard, S. A., Dunn, M. F., Luisi, P. L., & Shack, P. (1970) *Biochemistry 9*, 185] to propose half-of-the-sites reactivity for this enzyme. Fluorine magnetic resonance demonstrates that under equilibrium conditions at 4 °C and pH 8.75 *p*-(trifluoromethyl)benzyl alcohol binds to LADH with a dissociation constant of 1×10^{-3} M. Addition of 1 equiv of NAD to a stoichiometric complex of LADH and the alcohol generates a discrete resonance. This resonance corresponds to the ternary complex of LADH, NAD, and alcohol with little contribution from the NADH and aldehyde ternary complex. Quantitation shows that the bound NAD–alcohol pair is favored by at least a factor of 20 over the bound NADH–aldehyde pair at equilibrium. These results are consistent with explanations for the biphasic kinetics which involve half-of-the-sites reactivity of multiple steps in the reaction of each catalytic site. These results eliminate explanations for the biphasic kinetics of this system which require this equilibrium constant to have a value near unity or those which give greatest thermodynamic stability to the heterodimer LADH [(NAD·alcohol)(NADH·aldehyde)].

Alcohol dehydrogenase from horse liver (LADH) is an oligomeric enzyme composed of two 42 000 molecular weight subunits of identical amino acid sequence (Jörnvall, 1970) and four zinc atoms per dimer (Åkeson, 1964). The enzyme catalyzes the equilibrium

$$NADH + H^+ + RCHO \rightleftarrows NAD^+ + RCH_2OH$$

for a wide variety of alcohols and aldehydes (see Sund & Theorell, 1963, for a review) and has been thought to possess a compulsory-ordered reaction mechanism [Wratten & Cleland, 1963, 1965; see Brändén et al. (1975) for a review]. The enzyme will accept a wide variety of alcohol/aldehyde pairs as substrates. This rather extended specificity for substrates may be exploited by using various aromatic substrates in order to study the enzyme's kinetic behavior (Bernhard et al., 1970; Dunn & Bernhard, 1971; McFarland & Bernhard, 1972). Initial stopped-flow studies revealed biphasic kinetics with aromatic aldehydes as substrates and were extended to include aromatic alcohols reacting in the reverse direction (Luisi & Favilla, 1972; Luisi & Bignetti, 1974). The amplitudes of the fast and slow components of the time course were approximately equal when equal numbers of enzyme sites and substrates were present. These studies lead to the proposal that the enzyme possessed half-site reactivity with respect to substrates in either reaction direction with half of the sites showing the rapid kinetic behavior and half the slow behavior.

This possibility has become the subject of considerable controversy in recent years. Further reports have supported the existence of half-sited behavior (Baici & Luisi, 1977) and subunit interactions in this system (Lindman et al., 1972; Dunn et al., 1979). Other reports have presented biphasic kinetic data departing from a half-sited burst, offering alternate explanations for this behavior (Pettersson, 1976; Kvassmann & Pettersson, 1976; Weidig et al., 1977; Kordal & Parsons, 1979). In order to address some of the questions concerning half-site reactivity and/or subunit interactions in this system, we have employed equilibrium ^{19}F NMR measurements with ^{19}F-labeled substrates.

These substrates are similar to various para-substituted benzaldehydes and benzyl alcohols already widely used for investigation of this enzyme's mechanism [for example, see Wratten & Cleland (1965) or Dunn et al. (1979)]. The fluorinated aldehyde is shown here to exhibit biphasic kinetics in single-turnover experiments very similar to those published with other benzaldehyde derivatives (Bernhard et al., 1970).

By examining enzyme complexes at equilibrium, it should be possible to examine the plausibility of mechanisms relating to the purported half-site reactivity in this enzyme. In particular, a value near unity for the equilibrium constant between the ternary complexes

$$E(NAD\cdot alc)_2 \overset{K_{eq} = 1}{\rightleftharpoons} E(NADH\cdot ald)_2$$

could account for any observed biphasicity with roughly equal amplitudes [see discussion in Luisi & Bignetti (1974)]. Equivalently, the fully liganded heterodimer E(NADH·ald)(NAD·alc) could be a stable intermediate species on the reaction pathway such that reaction proceeds to this complex and does not proceed further until substrate or coenzyme dissociates from the complex. Although it has been suggested from stopped-flow data that the equilibrium between the central ternary complexes lies in favor of E·NAD·alcohol by 25-fold in the case of benzyl alcohol (Plapp et al., 1978), it should be of interest to directly determine the relative stability and identity of ternary complexes at equilibrium. Comparison

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of stabilities with those inferred from X-ray diffraction work (Plapp et al., 1978) should also be of interest.

This paper demonstrates that the ^{19}F-labeled substrates give distinct chemical shifts for free and bound alcohol, free and bound aldehyde, free aldehyde hydrate, the ternary complex of NAD, alcohol, and enzyme, and the abortive ternary complex of enzyme, NADH, and alcohol. Using these signals in conjunction with UV measurements of NADH production, we conclude that the equilibrium of the bound NAD·alcohol pair as compared to bound NADH·aldehyde lies at least 20-fold in the direction favoring the alcohol. This result rules out explanations for the biphasic kinetics observed for this system which invoke a slow equilibration between chemically distinct bound substrates with an equilibrium constant for that step near unity.

Materials and Methods

The LADH used in this paper was prepared and stored according to Anderson & Dahlquist (1979). This procedure gives pure EE isozyme of very high specific activity when assayed according to Dalziel (1957). The specific activity in pH 8.75 pyrophosphate buffer remained unchanged for periods of time well exceeding the duration of the NMR experiments reported in this paper. Throughout this paper, enzyme site concentration is expressed in normality, to distinguish it from dimer concentration.

Stopped-flow experiments were carried out in the laboratory of Dr. Michael Dunn at the University of California at Riverside, by use of a Durrum-Gibson D-110 stopped-flow spectrophotometer described in Dunn et al. (1979). A dead time of 3.0 ms was used. Data were collected at 328 nm, the isosbestic point of free and bound NADH, and fitted to one or two single exponentials by using a nonlinear least-squares program described in Dunn et al. (1979).

Some ^{19}F NMR spectra were collected on a Varian XL-100 NMR multinuclear spectrometer with a variable-temperature controller equipped for Fourier transform NMR and operating at 94.1 MHz for fluorine. The spectrometer was run in conjunction with a dedicated Varian 620/i computer with disk data storage. Some 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 as an internal chemical shift and field homogeneity standard. All reported chemical shifts are downfield from internal trifluoroacetate. When large changes in solution pH or solvent composition were made, chemical shifts were also compared with neat trifluoroacetic acid contained in a sealed glass capillary mounted concentrically inside the NMR tube. The trifluoroacetic acid gave a sharp resonance approximately 3.2 ppm upfield from that of aqueous trifluoroacetate. The chemical shift of observed resonances was then converted to positions relative to trifluoroacetate anion. No evidence of trifluoroacetate binding to LADH was observed under any experimental conditions at the concentrations used in all experiments, usually ca. 0.1 mM. The 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.

p-(Trifluoromethyl)benzaldehyde was obtained from PCR Research Chemicals Inc. (Gainesville, FL), vacuum distilled at room temperature over sodium bicarbonate, and stored frozen at −20 °C. Aqueous stocks were made up as necessary and stored at 4 °C. The aldehyde was checked for purity by both proton and fluorine NMR and was found to be stable for several months in tightly stoppered 4 °C aqueous solutions. The UV spectrum of the aldehyde in water shows a main peak at 286 nm with a molar extinction coefficient of 1730.

p-(Trifluoromethyl)benzyl alcohol was synthesized from the aldehyde by reduction with 98% sodium borohydride (Alfa Products, Danvers, MA). Typically, 1 mL of neat aldehyde was dissolved in 10 mL of anhydrous methanol, and 0.3 g of sodium borohydride was slowly added at room temperature with stirring. This was followed by the addition of 4 mL of water and 5 mL of 1 N sodium hydroxide, and then the mixture was refluxed for 15 min. The alcohol was extracted with methylene chloride, dried over magnesium sulfate, and filtered, and the methylene chloride was vacuum distilled. The alcohol was vacuum transferred to a storage vial. The purity of the alcohol was confirmed by proton and fluorine NMR. Its UV spectrum in water consists of peaks at 258, 263, and 269 nm with molar extinction coefficients of 409, 469, and 385, respectively.

The molar extinction coefficient of the alcohol was determined with two different methods. A known concentration of TFA was determined by titration with the NBS primary standard 187a $Na_2B_4O_7 \cdot 10H_2O$ as well as with an NaOH solution made from a JT Baker Dilut-it standard. This was added to an NMR tube with alcohol of known UV absorbance, giving a similar ^{19}F NMR peak height. A spectrum was collected with a delay between pulses of approximately five T_1 intervals ($T_1 \sim 2.5$ s for free TFA and alcohol at 25 °C), and relative peak areas were compared by integration of the peak intensities. The second method was to weigh a known volume of the alcohol, dilute in aqueous ethanol solutions, and obtain the UV spectrum. Extinctions obtained by extrapolation to zero ethanol concentration gave results identical with those from the NMR method above. Crystalline *p*-carboxybenzotrifluoride (PCR, Inc.) was used without further purification and gave a single ^{19}F NMR resonance. Grade III β-NAD and β-NADH were from Sigma. β-NADD used in stopped-flow experiments was a generous gift from Dr. Mike Dunn.

Binding constants were determined from fast-exchange NMR data according to an equation derived from Dahlquist & Raftery (1968). A nonlinear least-squares program was written in BASIC to determine best-fit values of K_s, the ligand dissociation constant, and Δ, the chemical shift (relative to free ligand) of ligand when fully bound. Initially, binding data were fitted to an approximate linearized equation (eq 1)

$$S_0 = (E_0\Delta)\frac{1}{\delta} - K_s \tag{1}$$

(derived from Dahlquist & Raftery, 1968) where E_0 = enzyme site concentration (kept constant as ligand is added to enzyme) and S_0 = total ligand concentration for each value of δ, the observed chemical shift relative to free ligand. The values obtained for K_s and Δ were employed as a first approximation in a least-squares fit to the complete form of the binding equation (eq 2). The values of K_s and Δ were varied around

$$S_0 = \frac{E_0\Delta}{\delta} - \frac{K_s}{1 - \frac{\delta}{\Delta}} \tag{2}$$

their linear best-fit values until a minimum summed squares value for each was found to a resolution of 1 Hz in Δ and 0.1 mM in K_s.

Ligand binding was also monitored by spin–lattice relaxation measurements. The determination of K_d is entirely analogous

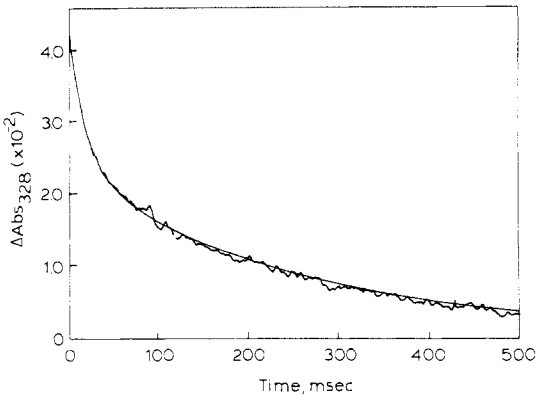

FIGURE 1: A representative stopped-flow trace of the LADH-catalyzed reaction of 20 μN enzyme in one syringe with 19.4 μM NADD and 1.35 mM *p*-(trifluoromethyl)benzaldehyde in the second syringe. Parameters for this trace derived from a nonlinear least-squares fit to two exponentials plus A_∞ are $k_{fast} = 50.2\ s^{-1}$, $k_{slow} = 4.45\ s^{-1}$, and $A_{fast}/(A_{fast} + A_{slow}) = 0.52$ after correction for an instrument dead time of 3 ms. The theoretical best-fit (smooth) line is overlaid on the data. Duration of the trace is 0.5 s. A single exponential was found to be inadequate to fit the data.

to the above procedure under fast-exchange conditions, where $\delta = 1/T_{1,obsd} - 1/T_{1,free}$ and $\Delta = 1/T_{1,bound} - 1/T_{1,free}$ [see Dwek (1973) for example].

Spin-lattice relaxation times were determined by standard inversion recovery methods.

Results

Biphasicity of p-$CF_3C_6H_4CHO$ in Stopped-Flow Studies. Figure 1 shows a stopped-flow kinetic trace at 328 nm which results when 20 μN enzyme is mixed with 19.4 μM NADD and 1.35 mM *p*-$CF_3C_6H_4CHO$. The deuterated derivative, NADD, is used in place of NADH in order to slow the rate of the fast step, which has been shown by McFarland & Bernhard (1972) and Jacobs et al. (1974) to be subject to an isotope effect of approximately 2–3. Only a single turnover of enzyme sites is expected since NADD is slightly substoichiometric to enzyme site concentration. The trace was found not to fit a single exponential time course, but could be well-described by two exponentials of the form

$$A(t) = A_1e^{-k_1t} + A_2e^{-k_2t} + A_3$$

where $A(t)$ is the observed amplitude vs. time. The parameters A_1, k_1, A_2, k_2, and A_3 are phenomenological constants used to give the best fit. Computer-assisted nonlinear least-squares analysis by use of the double exponential scheme for eight separate experiments gave the following values: $k_1 = 49 \pm 7.5$, $k_2 = 4.7 \pm 2.4$, and $A_1/(A_1 + A_2) = 0.52 \pm 0.03$.

The amplitude of the fast phase constitutes 52% of the summed fast plus slow-phase amplitudes under these conditions. While more stopped-flow data with this substrate are necessary to address the question of a half-sited function for LADH with this method, the kinetic trace is reproducibly biphasic under these conditions and similar to data obtained in experiments with benzaldehyde as a substrate (Bernhard et al., 1970).

Free-Ligand ^{19}F NMR Spectra. Fluorine magnetic resonance spectroscopy demonstrates that *p*-(trifluoromethyl)-benzyl alcohol and the analogous aldehyde and acid each possess a unique chemical shift: free alcohol resonates at 13.40 ppm downfield from the internal standard TFA, free acid at 13.06 ppm, and free aldehyde at 12.46 ppm. Spectra of aqueous solutions of aldehyde show a small peak at 13.20 ppm which is 8–10% of the height of the main peak.

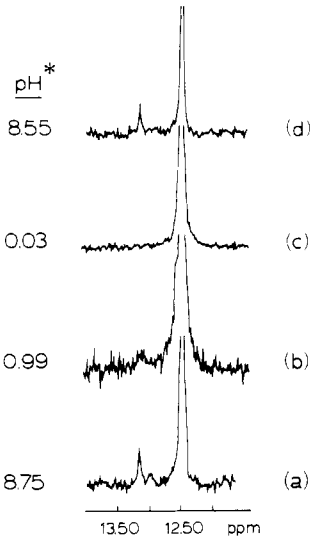

FIGURE 2: The behavior of the two ^{19}F NMR resonances observed in a 4.5 mM aqueous solution of *p*-(trifluoromethyl)benzaldehyde at 20 °C as a function of pH* (see Materials and Methods). The pH* value is decreased by addition of 6 N HCl from a pH* of 8.75 (a) to 0.99 (b), 0.03 (c), and back to 8.55 (d) with aqueous NaOH. A total of 6000 free induction decays (FIDs) were averaged for (a) and (b), 4000 for (c) and (d).

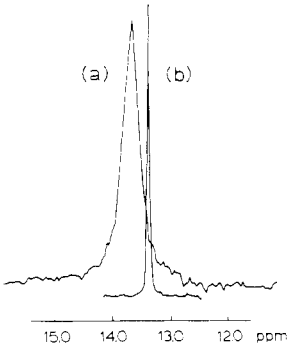

FIGURE 3: Binary complex of LADH with *p*-(trifluoromethyl)benzyl alcohol as shown by ^{19}F NMR. The upfield line at 13.40 ppm belongs to free alcohol, at pH 8.75 and 20 °C. Superimposed for comparison is a downfield resonance at 13.71 ppm which is due to a fast-exchange average of free and bound alcohol. LADH (0.65 mN) and alcohol (0.65 mM) were present at pH 8.75, 20 °C, in 50 mM sodium pyrophosphate buffer. A total of 36 000 transients were accumulated for the downfield peak.

Figure 2 shows the pH dependence of the ^{19}F spectrum of an aqueous solution of *p*-(trifluoromethyl)benzaldehyde in the region of the 13.20-ppm line. As proton concentration increases, the line broadens perceptibly until it has disappeared at pH* 0.03. The 13.20-ppm peak reappears when the pH* is raised back to 8.55. This behavior is exactly that expected for a reversible acid-catalyzed increase in the rate of hydration of aldehyde (Bell, 1966) which results in a broadening of the hydrate resonance and its eventual averaging with the aldehyde resonance as the exchange rate of the hydrate with the aldehyde increases. The chemical shift and line width of the aldehyde line are not visibly affected since its population of spins is much larger than that of the 13.20-ppm resonance.

The small peak at 13.20 ppm is seen to decrease in intensity as dioxane is added to the solution. If the line were due to an impurity different from hydrate, it would seem likely that its signal should remain present in dioxane. At 80:20 dioxane/water (v/v), the resonance is greatly diminished, and it disappears by 95:5 dioxane/water. In addition, spectra run with neat aldehyde do not show the resonance at 13.20 ppm downfield, reinforcing its assignment to the hydrated aldehyde.

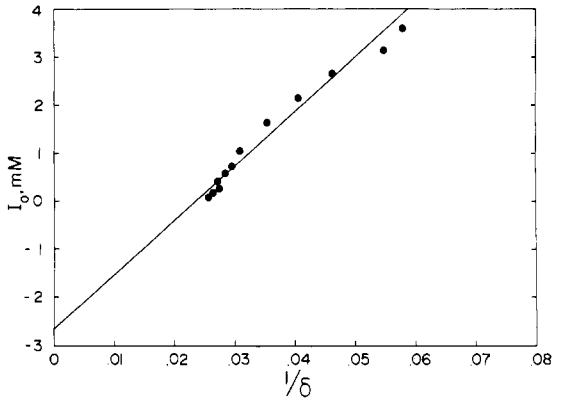

FIGURE 4: Determination of the binding constant of *p*-(trifluoromethyl)benzyl alcohol to LADH by plotting $[alcohol]_{total}$ vs. $1/\delta$. The best-fit nonlinear curve was determined by a function-minimization procedure as described under Materials and Methods. The best-fit line is shown. The derived K_d in pH 8.75 50 mM sodium pyrophosphate buffer at 4 °C is 2.67 mM for the alcohol.

Binary Enzyme Complexes with Alcohol and Acid. Figure 3 presents a spectrum of the binary complex of enzyme with alcohol under conditions where alcohol is slightly substoichiometric to enzyme sites. The peak has shifted approximately 0.36 ppm downfield from the *free* alcohol peak, which occurs at 13.39 ppm from TFA. As more alcohol is added to enzyme, only a single peak appears, shifting more toward the free alcohol position as more alcohol is added. Thus, the observed line represents a fast-exchange average of the free and bound environments.

Under these conditions, the alcohol concentration dependence of the observed chemical shift of the alcohol, δ, from its free position may be employed to obtain both the dissociation constant, K_s, and the shift of the bound environment from the free, Δ, according to Dahlquist & Raftery (1968). Figure 4 shows a plot of $[alcohol]_{total}$ vs. $1/\delta$ for a typical titration. The calculated K_d was 2.67×10^{-3} M at pH 8.6 and 4 °C. The bound shift was 1.71 ppm downfield from the free position. For four separate experiments, an average K_d of 2.05 mM, with a range from 1.49 to 2.67 mM, was obtained. An average bound shift for alcohol of 1.73 ppm, with a range from 1.46 to 1.98 ppm, was found. Two determinations of K_d by T_1 measurements yielded an average value of 1.73 mM.

It should be noted that the chemical shift of "free" alcohol used is not necessarily that of alcohol in the absence of enzyme. It must be the shift of alcohol in the presence of enzyme but without any contribution due to binding. This was determined in two ways. First, enzyme active sites were occupied with the potent inhibitors NAD·pyrazole. At 4 °C, pH 8.6, in the presence of 0.2 mN enzyme, 0.3 mM NAD, and 0.3 mM pyrazole, the shift of 0.2 mM alcohol was found to be 13.440 ppm from TFA. Second, at very high ratios of alcohol to enzyme, only a tiny fraction of the total alcohol is bound. Thus, the shift is due to alcohol in the presence of enzyme, but in the absence of a significant contribution due to bound alcohol. With a 27.7-fold excess of alcohol (0.199 mN enzyme, 5.52 mM alcohol), the shift was 13.436 ppm.

The presence of LADH also affects the chemical shift of *p*-(trifluoromethyl)benzoic acid. A single fast-exchange average resonance is observed under all conditions. Since the enzyme causes a large shift (some 0.67 ppm) under the conditions of this experiment, the shift in peak position is more readily visualized than in the case of the alcohol. The concentration dependence of the observed shift, shown in Figure 5, allows calculation of the dissociation constant to be 0.50 mM, and the bound shift 1.25 ppm. Thus, the fully bound

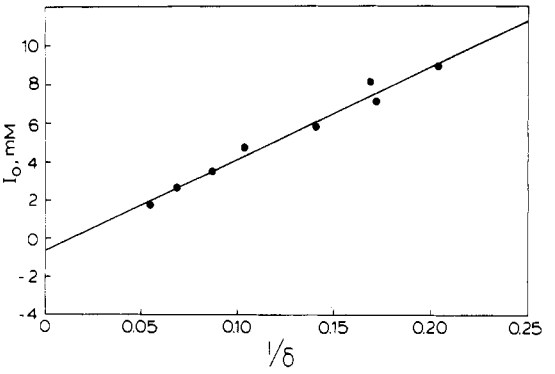

FIGURE 5: Determination of best-fit K_d for *p*-(trifluoromethyl)benzoic acid of 0.5 mM (pH 8.75, 20 °C).

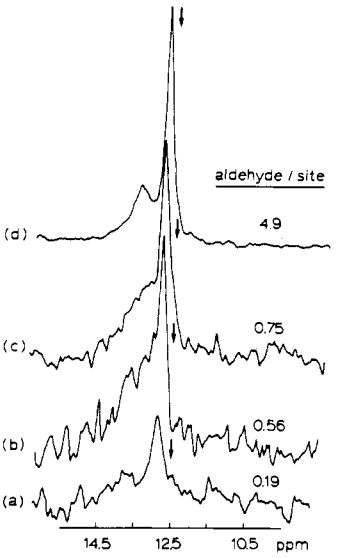

FIGURE 6: Titration of enzyme with *p*-(trifluoromethyl)benzaldehyde. Shown are the two ^{19}F NMR lines observed when aldehyde is added to enzyme. The upfield resonance is fast exchange with free aldehyde, whose position is marked by an arrow at 12.47 ppm from TFA. The ratio of total aldehyde to enzyme sites it listed at the right of each spectrum. Parameters for each spectrum are as follows: (a) peak is 12.85 ppm from TFA, with a peak width at half height ($\Delta\nu_{1/2}$) of 34 Hz; 10000 FIDs were averaged; (b) 12.78 ppm, $\Delta\nu_{1/2}$ = 32 Hz, 3000 FIDs averaged; (c) 12.76 ppm, $\Delta\nu_{1/2}$ = 24 Hz, 2000 FIDs; (d) 12.66 ppm, $\Delta\nu_{1/2}$ = 21 Hz, 1000 FIDs. Enzyme site concentration in (a–d) was approximately 0.45 mN at pH* 8.75, 4 °C.

acid should appear at 14.31 ppm downfield from TFA.

Binary Enzyme·Aldehyde Complexes. Binary complexes with aldehyde are observable as well (Figure 6). Unlike the case for alcohol and acid, two lines are observed when aldehyde is added to enzyme. Neither is coincident with free aldehyde, which comes 12.52 ppm downfield from TFA. The upfield line is in fast exchange with free aldehyde since it moves toward free aldehyde, and its width at half-maximum amplitude narrows as more aldehyde is added. The downfield line not only is much broader but also appears to be in slow exchange or to have no exchange with free aldehyde. As additional aldehyde is added, it appears to coalesce to a position about 13.37 ppm downfield from TFA. From its fast-exchange averaging with free aldehyde, the upfield line can be postulated to be enzyme-bound aldehyde in a binary complex.

The downfield line disappears when the solution of enzyme and aldehyde in Figure 6 is made 3% w/v in $NaDodSO_4$, which denatures the enzyme.

Ternary Enzyme·NAD·Alcohol Complex. Figure 7 shows a stoichiometric titration of binary enzyme·alcohol complex with NAD. Spectrum a with no NAD added shows the single

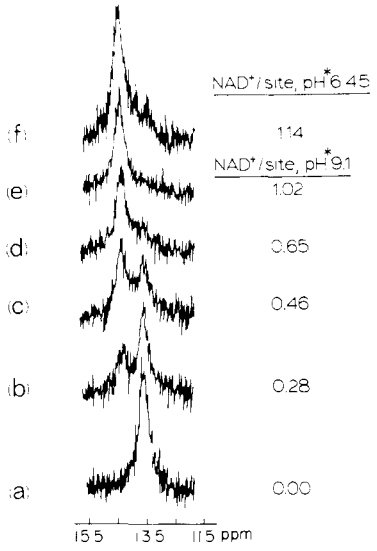

FIGURE 7: ^{19}F NMR titration of binary fast-exchange enzyme·*p*-(trifluoromethyl)benzyl alcohol complex with NAD. The ratio of alcohols/site was 0.75. NAD/site was varied as shown beside each spectrum. (a) The single line of the binary enzyme·alcohol complex 13.74 ppm downfield from trifluoroacetate. No NAD has been added. (b) 0.28 NAD/site; resonances appear at 13.75 and 14.43 ppm. (c) 0.46 NAD/site; resonances at 13.69 and 14.41 ppm. (d) 0.65 NAD/site, resonance at 14.45 ppm. A total of 5700 FIDs were averaged for each spectrum. Enzyme site concentration was constant at 0.56 mN at pH* 9.1, 4 °C. (f) Spectrum equivalent to (e) except at pH* 6.45, 4 °C; [enzyme sites] = 0.704 mN with 0.95 alcohols/site, 1.14 NAD/site. Chemical shift of peak is 14.45 ppm from trifluoroacetate, as in (e). A total of 4000 FIDs of 0.8-s duration were averaged.

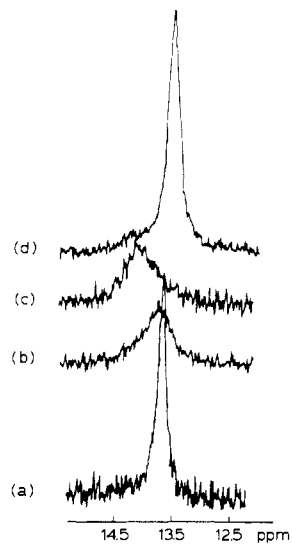

FIGURE 8: Formation of an abortive ternary complex of enzyme, NADH, and *p*-(trifluoromethyl)benzyl alcohol observed by ^{19}F NMR. (a) A typical fast-exchange average line between enzyme and alcohol. No NADH has been added yet. There is 1.0 alcohol/site with the lone resonance appearing 13.69 ppm from trifluoroacetate. (b) Spectrum taken after the addition of 0.40 NADH/site to the binary enzyme·alcohol complex. The peak now appears at 13.84 ppm. (c) Addition of NADH to 0.81 NADH/site. The resonance is at 14.16 ppm. (d) Addition of excess alcohol to (c). Enzyme sites/NADH/alcohol ratio is 1.0:0.81:3.8. The peak is shifted to 13.54 ppm from trifluoroacetate. The enzyme concentration for (a–d) is 0.450 mN at pH* 8.75, 4 °C. A total of 5500 FIDs were averaged for each spectrum.

peak expected of the binary complex in fast exchange with free alcohol at 13.71 ppm from TFA. As NAD is added in subsequent spectra (b–e), it can be seen that a new line downfield from the binary complex line begins to form at 14.40 ppm as more and more NAD is added. It forms at the expense of the 13.71-ppm line, which decreases in amplitude as the new line increases. The new line represents another environment for the alcohol, which is being created as NAD is being added to the enzyme·alcohol complex. When the total concentration of added NAD equals the total concentration of added alcohol, the binary complex peak has disappeared, and the new peak, still at 14.40 ppm, is the only peak remaining. This behavior is that expected of a stoichiometric conversion of the enzyme·alcohol binary complex to the enzyme·NAD·alcohol ternary complex by addition of NAD. The binary enzyme·alcohol complex and the ternary enzyme·NAD·alcohol complex are in slow exchange on the NMR time scale. Since NAD binds to enzyme at pH 8.75 with a K_d of 12–20 μM (Taniguchi et al., 1967; Weidig et al., 1977), enzyme might also be expected to be present mainly as ternary complex. Of course, NAD and alcohol can react to form NADH and aldehyde. However, no aldehyde is observed by NMR. The peak at 14.40 ppm also results when stoichiometric NADH, aldehyde, and enzyme are observed.

In addition, the UV spectrum of the contents of the NMR tube immediately after the last spectrum in Figure 7 was determined. Very little absorption was found at 330 mm, the isosbestic point of bound and free NADH. When an analogous titration to Figure 7 was done by UV spectrometry instead of NMR, approximately 5% of the initially added NAD was present as NADH, after the blank reaction (see Taniguchi, 1967, for example) was allowed to equilibrate.

Abortive Ternary Complexes. We have succeeded in producing the abortive ternary complex E·NADH·alcohol (Figure 8), which has been hypothesized to exist in the mechanism for the enzyme proposed by Hanes et al. (1972) and Dalziel & Dickinson (1965, 1966). Figure 8 shows the spectra which result when NADH is added to the binary enzyme·alcohol complex. The binary alcohol line is shifted downfield toward 14.40 ppm with the addition of NADH. Unlike the case for the E·NAD·alcohol complex, ternary alcohol in E·NADH·alcohol is in intermediate for fast exchange with binary and free alcohol, so that only one line of average position between fully bound abortive ternary, binary, and free alcohol is observed. Thus, as more alcohol is added, the averaged resonance shifts upfield toward free alcohol.

We have also attempted to produce the abortive E·NAD·aldehyde complex. However, when NAD and aldehyde are mixed with enzyme, a dismutation of aldehyde occurs to produce the E·NAD·alcohol line at 14.40 ppm and a line due to *p*-(trifluoromethyl)benzoic acid at 13.04 ppm, as shown in Figure 9a. The identity of the 14.40-ppm peak can be confirmed as alcohol by addition of 1 mL of 10% $NaDodSO_4$ and subsequent observation of the spectrum. As shown in Figure 9b, two narrow peaks are observed. One of these is 13.24 ppm downfield from TFA and is identical in position with a standard of alcohol in a $NaDodSO_4$ solution, while the second line 13.10 ppm downfield from TFA is identical in shift with a standard of *p*-(trifluoromethyl)benzoic acid in the same $NaDodSO_4$ solution. Thus, it appears that the aldehyde is dismutated into alcohol and acid in the presence of LADH and NAD. This dismutase-like activity has been found with other LADH substrates as well (Kendal & Ramanathan, 1952; Abeles & Lee, 1960; Dalziel & Dickinson, 1965; Gupta, 1970; Hinson & Neal, 1972, 1975).

Discussion

In this paper, we introduce a novel method for the observation of bound alcohol dehydrogenase complexes at equilib-

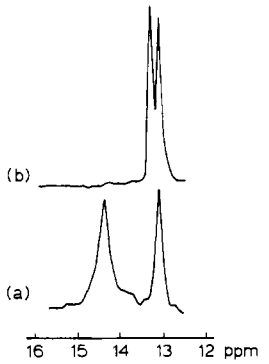

FIGURE 9: The occurrence of a dismutase-like reaction upon attempted ^{19}F NMR observation of an abortive ternary complex of enzyme, NAD, and aldehyde. (a) 0.375 mN enzyme incubated at pH* 8.75, 20 °C, with 0.56 mM *p*-(trifluoromethyl)benzaldehyde and 0.412 mM NAD. The spectrum consists of an average of 4000 FIDs. Resonances are at 14.38 and 13.10 ppm from TFA. Both spectra are apodized to improve the signal/noise ratio. (b) Spectrum after addition of $NaDodSO_4$ solution to 3% w/v, giving resonances at 13.24 and 13.10 ppm which correspond exactly to standards of alcohol and in 3% $NaDodSO_4$ under these conditions.

Table I: Chemical Shifts of ADH Ligands[a]

ligand	free shift	shift upon binding (ppm) binary complex	ternary complex
alcohol	13.40	1.73	1.05,[b] >0.73[c]
aldehyde	12.47		
aldehyde hydrate	13.20		
acid	13.04	1.34	

[a] All shifts are in parts per million downfield from the internal standard trifluoroacetic acid anion dissolved in the aqueous buffer. [b] Ternary complex with NAD. [c] Ternary complex with NADH.

rium. By using ^{19}F NMR, we can observe separate lines for free and bound *p*-(trifluoromethyl)benzyl alcohol, aldehyde, and acid, hydrated aldehyde, the productive ternary complex between enzyme, NAD, and alcohol, and the abortive ternary complex of enzyme, NADH, and alcohol. Table I summarizes the various chemical shifts corresponding to these species.

Since LADH has an extended specificity with regard to substrate structure, our fluorinated alcohol and aldehyde are active substrates (Figure 1). In fact, in the stopped-flow experiments designed to look at aldehyde reduction, the partitioning of the amplitude of reaction into a fast and slow phase closely resembles that for benzaldehyde reported previously under similar experimental conditions (Bernhard et al., 1970). Benzaldehyde, benzyl alcohol, and various para-substituted derivatives have been extensively used in studies investigating aspects of the LADH reaction mechanism (Dunn et al., 1979; Plapp et al., 1978; Kordal & Parsons, 1979; Kvassman & Pettersson, 1976, 1978; Weidig et al., 1977; Baici & Luisi, 1977; Dworschack & Plapp, 1977; Tatemoto, 1975a,b; McFarland & Chu, 1975; Jacobs et al., 1974; Dunn, 1974; Luisi & Bignetti, 1974; McFarland et al., 1974; Luisi & Favilla, 1972; McFarland & Bernhard, 1972; Bernhard et al., 1970; Wratten & Cleland, 1965). It should be apparent that our substituted benzaldehyde and benzyl alcohol are not unusual compounds for studying this problem. Using the pure E isozyme of LADH, we have found that we can easily observe binary complexes of the enzyme with our fluorinated alcohol, aldehyde, and acid.

In each case, a substantial downfield shift of the fluorine resonance associated with these ligands is observed in the presence of LADH. The alcohol and acid resonances show only single lines in the presence of the protein, which shift as a function of the protein and ligand concentration. This suggests rapid exchange of these ligands on and off the protein surface. As shown in Table I, the calculated bound shift of the alcohol in its binary comlex is 1.7 ppm, and the bound shift of the acid is 1.3 ppm downfield of their free positions. It is interesting to compare these shifts to that observed in the ternary complex of LADH·alcohol·NAD of 1.05 ppm downfield of the free ligand chemical shift. The similarities of the shift on binding of these binary and ternary complexes of the alcohol suggest that the alcohol and acid are bound in a similar environment.

The interaction of the aldehyde with the protein is more complex. In the presence of LADH, the aldehyde shows two resonances, a relatively sharp line somewhat downfield of the chemical shift of the free aldehyde and a second much broader resonance further downfield. The sharper line represents a fast-exchange average of bound aldehyde with free. The broader line is more difficult to assign. Only a single resonance is observed after denaturing the protein, so no net chemical reaction such as aldehyde disproportionation has occurred. Thus, is appears that this resonance must represent a reversible but more slowly exchanging association of aldehyde and protein. One possibility is that this represents aldehyde which has undergone reaction with lysine residue(s) to form the Schiff base product.

The existence of these binary complexes rules out any mechanism for aromatic substrates which does not allow for the existence of binary enzyme complexes with substrates but does not comment on which pathway (NAD addition to enzyme before addition of alcohol, or vice versa) might be kinetically preferred.

Our evidence for the existence of the E·NADH·alcohol abortive ternary complex also supports the proposals by Wratten & Cleland (1965), Dalziel & Dickinson (1966a,b), Hanes et al. (1972), and Baici & Luisi (1977) for its existence. Under the conditions we have employed, we cannot observe the E·NAD·aldehyde complex since it disproportionates to form acid and alcohol within the 0.5-h mixing required to obtain the ^{19}F spectrum.

Mixtures of enzyme, coenzyme, and substrate must not be allowed to stand "at equilibrium" for too long (usually greater than 12 h), depending on temperature and the ligands present. As is evident from ^{19}F NMR measurements made over a long period of time, there is a slow evolution of acid and NADH on a time scale of many hours. This seems to be due to further oxidation of the small amount of aldehyde produced by the normal reaction [see, for example, Hinson & Neal (1975)]. This would explain the observation by Plapp et al. (1978) of substantial NADH production in crystals of enzyme, NAD, and *p*-bromobenzyl alcohol after 6 months. To further complicate matters, when high (several millimolar) NAD is incubated with enzyme, with no other substrate, a "blank" reaction (see Taniguchi et al., 1967; Taniguchi, 1967; Luisi & Bignetti, 1974) occurs, which appears to produce NADH. Because of these complications, we found it necessary to accumulate spectra at 4 °C to slow these side reactions. The presence of an easily detected acid resonance (no other sharp substrate peaks are assignable to the range between 13.39 and 13.04 ppm in aqueous buffer solutions) serves as a signal that significant side reactions have occurred.

When 1 equiv each of enzyme, NAD, and fluorinated alcohol is mixed at concentrations in the range of 10^{-4} M, a single ^{19}F NMR resonance is observed, 14.40 ppm downfield from TFA, which exchanges slowly with superstoichiometric

alcohol. This resonance does not correspond to the binary complexes of enzyme formed with alcohol or aldehyde and must therefore represent a ternary complex. Since the alcohol and NAD can react to form aldehyde and NADH, the final equilibrium distribution observed will represent the most thermodynamically stable mixture of the four possible ternary complexes. We have investigated the two abortive ternary complexes of NADH·alcohol and NAD·aldehyde in separate experiments. The complex formed with NADH and alcohol shows fast exchange with superstoichiometric alcohol (Figure 8); thus, it cannot correspond to the observed resonance. When NAD and aldehyde are mixed in the presence of LADH, an immediate disproportionation reaction is observed (Figure 9), and for each 2 mol of aldehyde one each of alcohol and acid is formed. Since we see only a very slow production of acid when LADH, NAD, and alcohol are mixed, the single line observed cannot represent the abortive complex formed with aldehyde and NAD.

Thus, it is clear that the single line we observed when LADH, alcohol, and NAD are mixed represents the equilibrium distribution of the reactive ternary complexes E·NAD·alcohol and E·NADH·aldehyde. As expected, this same resonance is observed if 1 equiv each of LADH, NADH, and aldehyde is mixed, showing that the system is at equilibrium.

We have estimated the value of the equilibrium constant between the two ternary complexes by measuring the NADH produced from its UV absorption at 330 nm. These measurements were performed on exactly the same samples used for the NMR experiments, again with an initial mixture of 1 equiv each of enzyme, alcohol, and NAD. This measurement demonstrates that less than 5% of the NAD present in the original mixture has been converted to NADH.

Thus, the equilibrium constant between the reactive ternary complexes must favor the E·NAD·alcohol complex by at least a factor of 20. The single line observed at 14.40 ppm can therefore be assigned to the E·NAD·alcohol complex with no more than a 5% contribution from the E·NADH·aldehyde complex. The resonance corresponding to this less favored complex may exist as a separate undetected line. Alternatively, the hydride transfer step may be fast enough to cause rapid exchange averaging of the two reactive ternary complexes to give a single composite line.

Our conclusion is supported by the work of Plapp et al. (1978). From kinetic considerations with benzyl alcohol and benzaldehyde, they concluded that the E·NAD·benzyl alcohol ternary complex should predominate 28-fold over E·NADH·benzaldehyde. This work assumed no complications arising from half-of-the-sites reactivity or subunit interaction so that the bound equilibria could be estimated directly as a ratio of the observed hydride transfer rates in both directions. It is interesting that this simple kinetic approach gives smaller values to our direct measurements. Of course, our experiments cannot be directly compared to their work since the electron-withdrawing p-CF_3 substituent could have a significant effect on the equilibrium constant.

It is interesting to compare the equilibrium constant for the free reactants and products to the values in the bound state. From studies on the effect of para substitution and of aromatic substrates on the solution equilibrium (Klinman, 1972; Dunn et al., 1979), the solution of the equilibrium

$$K = \frac{(p\text{-}CF_3 \text{ alcohol})(\text{NAD})}{(p\text{-}CF_3 \text{ aldehyde})(\text{NADH})}$$

gives a value of about 10^3 at pH 8.75 and 25 °C. We estimate the corresponding bound equilibrium given by

$$K' = \frac{(\text{bound } p\text{-}CF_3 \text{ alcohol})(\text{bound NAD})}{(\text{bound } p\text{-}CF_3 \text{ aldehyde})(\text{bound NADH})} = \left[\frac{\text{E·NAD·alcohol}}{\text{E·NADH·aldehyde}}\right]^2 \geq 400$$

This suggests that the bound and free apparent equilibria may be quite similar.

Implications for Putative Half-Sitedness of LADH. The determination that the equilibrium constant between E·NAD·alcohol and E·NADH·aldehyde is at least 20 has several consequences is restricting possibilities for the apparent half-sited reaction kinetics of this enzyme, which is currently in dispute in the literature. There is now a direct measurement of the equilibrium distribution of the two productive ternary complexes to rule out the argument that the biphasicity observed in the stopped flow results from an equilibrium constant of or near 1.0 between the two productive ternary complexes. Another similar hypothesis involving the enhanced stability of the mixed heterodimer E(NAD·alc)(NADH·ald) also appears to be ruled out by the same evidence.

We found no evidence for a second long-lived E·NAD·alcohol isomer at equilibrium. This rules out a variant of the $K_{eq} = 1$ hypothesis (Luisi & Bignetti, 1974) which involves an equilibrium constant of unity at this pH between two ternary E·NAD·alcohol complexes. Our results show that at least as far as the environment of the fluorine atoms of the substrates is concerned, there is only one type of ternary E·NAD·alcohol complex. If other complexes exist, they must interconvert rapidly with this complex, or they must also have a similar chemical shift to the major peak we observe.

Our results do not support the notion of differential behavior of the two sites upon NAD binding to E·alcohol. The chemical shift of the alcohol in the ternary complex remains invariant as more and more NAD is added, so the bound environment seems constant as a function of ligand saturation. Since the binding of NAD appears nearly stoichiometric throughout the titration of enzyme·alcohol with NAD, this eliminates any extreme negative cooperativity in the binding of NAD in the complex such that NAD binds only to one-half of the sites. Such strong negative cooperativity has been suggested to apply the E·NAD·pyrazole complex (McFarland & Bernhard, 1972).

Kvassman & Pettersson (1978) have obtained evidence for the dependence of the apparent rate of hydride transfer from benzyl alcohol to NAD on a group with $pK_a = 6.4$, which they have assigned to the coupled acid–base system of enzyme-bound alcohol, serine-48 and histidine-51.

At pH*'s 9.1 and 6.45, the shift of the resonance due to the ternary complex of enzyme, NAD, and alcohol is identical (Figure 7). It is expected that bound alcohol and alcoholate should occur at different chemical shifts. Thus, it seems likely that there is no measurable pK_a for p-(trifluoromethyl)benzyl alcohol within the ternary complex over this pH range.

In view of the electron-withdrawing character of the p-trifluoromethyl group (σ Hammett = 0.551; Hammett, 1970), it might be expected that the pK_a of bound alcohol or this proton-relay system would be even lower than that involving benzyl alcohol. Thus, our results are not necessarily in conflict with those of Kvassman & Pettersson, but give no indication for this substrate of a pK_a near pH 6.4 which affects the chemical shift of the bound alcohol.

These observations tend to rule out explanations for the biphasic kinetic behavior which involve equilibrations between the alcohol and alcoholate anion when bound to the ternary complex, since there is apparently only one dominant ionized species present.

Our NMR measurements have been made over a relatively long time scale (a minimum of 30 min). This may allow for the generation of thermodynamically stable, but nonproductive, complexes to be formed. We have preliminary evidence which suggests that the exchange of our observed ternary complex resonance of E·NAD·alcohol with free alcohol proceeds at a rate comparable to the normal rate of alcohol and/or NAD desorption in a normal kinetic assay. Thus, the complex we observe can desorb with normal kinetics. This suggests that it is along the normal kinetic path from reactants to products. Further work is under way to clarify this point.

While our observations rule out a number of possible explanations for the biphasic kinetic behavior of LADH observed with *p*-(trifluoromethyl)benzyl alcohol and the corresponding aldehyde, two major categories of explanations remain as viable alternatives. The first of these allows for either an induced or preexisting half-of-the-sites reactivity in which one subunit shows rapid kinetic behavior while the other site shows the slower time course. The second viable explanation supposes that the biphasic kinetics are intrinsic to a single subunit and represent a complex and as yet not fully understood chemical mechanism. Further publications from this laboratory will attempt to distinguish these possibilities.

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