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Fluorinated Ligands as Nuclear Magnetic Resonance Probes of Active-Site Nonequivalence in Abortive Ternary Complexes of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: The interactions of the dimeric horse liver alcohol dehydrogenase (LADH) with ligands in two nonreactive ternary complexes have been examined by using nuclear magnetic resonance and fluorescence techniques. One complex contains the enzyme, NADH, and the abortive alcohol substrate *p*-(trifluoromethyl)benzyl alcohol. The trifluoromethyl group allows the bound environment and bound to free exchange kinetics of this alcohol to be examined by using ^{19}F NMR. Binding isotherms of the coenzyme and the abortive substrate were examined by using fluorescence. Similar measurements were made with the enzyme, NADH, and the aldehyde analogue *p*-(trifluoromethyl)benzamide. For both complexes there was no evidence of cooperative equilibrium binding of any ligand. Careful measurements of the exchange kinetics of the fluorinated alcohol or amide when binding to the enzyme·NADH complex using NMR techniques showed that a single lifetime describes the exchange of ligands from both subunits of the protein. These results appear to rule out any site–site interactions in this system and support the notion that the observed biphasic kinetics observed in transient reactions of LADH with NAD and alcohols is a property of a single site in this system.

Horse liver alcohol dehydrogenase (LADH) is a dimeric zinc metalloenzyme which is one of the most thoroughly studied enzymes. However, the existence of intrasubunit communication or active-site nonequivalence within this dimer during catalysis has remained uncertain. Numerous reports have inferred half-of-the-sites reactivity (Bernhard et al., 1970; Dunn & Bernhard, 1971; McFarland & Bernhard, 1972; Luisi & Favilla, 1972; Luisi & Bignetti, 1974; Baici & Luisi, 1977; McFarland et al., 1977) or subunit interactions (Lindman et al., 1972; Dunn et al., 1979) within this system, while other reports either have found no evidence to support half-of-the-sites reactivity (Shore, 1969; Tatemoto, 1975; Hadorn et al., 1975) or have offered alternate explanations for data purporting to support a half-sited function for LADH (Pettersson, 1976; Kvassman & Pettersson, 1976; Weidig et al., 1977; Kordal & Parsons, 1979; Andersson & Mosbach, 1979).

We have recently examined half-inactivated LADH dimers for kinetic evidence of half-of-the-sites reactivity or subunit interactions. We also examined equilibrium binding isotherms and ligand desorption kinetics of LADH·NAD ternary complexes with trifluoroethanol or pyrazole for evidence of subunit interactions. These results were consistent with independent binding of ligands at each active site of the LADH dimer (Anderson & Dahlquist, 1982a,b).

In this report we have looked for equilibrium and kinetic evidence pertaining to subunit interactions in LADH by the study of complexes containing bound NADH in which turnover is not possible. Using ^{19}F NMR we are able to extend our earlier observations (Anderson & Dahlquist, 1980) and observe unique resonances for the substrate *p*-(trifluoromethyl)benzyl alcohol and the aldehyde analogue *p*-(trifluoromethyl)benzamide in complexes with LADH and NADH. Binding isotherms derived from ^{19}F NMR measurements and from protein NADH fluorescence quench measurements are consistent with independent ligand binding to each site of the dimer. We see only one type of bound environment in every LADH·NADH complex examined by ^{19}F NMR and only one desorption rate of bound *p*-(trifluoromethyl)benzamide. This suggests identical binding sites with identical ligand binding kinetics.

In competition experiments we have determined that the stability of alcohol in the LADH·NADH·alcohol complex is similar to that in the LADH·NAD·alcohol complex. This observation rules out mechanisms to explain the kinetic biphasicity observed during the corresponding aldehyde reduction that depend on an unusual kinetic or thermodynamic stability for this complex. Other mechanisms invoking unusual properties of LADH dimers with the abortive complex of NADH and alcohol bound at one site also appear inconsistent with our data.

Materials and Methods

Horse liver alcohol dehydrogenase was prepared as described in Anderson & Dahlquist (1979). Enzyme active site concentration was determined by the NAD–pyrazole assay of Theorell & Yonetani (1963) and is expressed in normality (*N*) to be distinguished from enzyme dimer concentration. β-NADH (grade III) was purchased from Sigma. *p*-(Trifluoromethyl)benzaldehyde was obtained from PCR, Inc.

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(Gainesville, FL). The synthesis of *p*-(trifluoromethyl)benzyl alcohol and purification of the corresponding aldehyde are described in Anderson & Dahlquist (1980). *p*-(Trifluoromethyl)benzamide was obtained from Marshalton Research Labs (Westchester, PA).

All ^{19}F NMR measurements were obtained on a Nicolet NTC-150 NMR spectrometer with variable temperature control operating at 141.184 MHz for fluorine, using 12-mm external diameter tubes. All binary complex titration data were obtained from the titration of LADH with ligands under fast-exchange conditions. Spin–spin relaxation measurements were obtained by the Hahn spin-echo technique. All chemical shifts are reported in ppm downfield from the internal standard trifluoroacetate (TFA). Peak areas were compared by using the product of their height times width at half-height, both of which were derived from the best-fit Lorentzian line using a program provided with the Nicolet data system.

Fluorescence Measurements. All fluorescence titrations were on a Schoeffel fluorometer as described in Anderson & Dahlquist (1982a). For NADH binding to LADH with *p*-(trifluoromethyl)benzyl alcohol, excitation was at 294 nm and emission was at 340 nm. For *p*-(trifluoromethyl)benzamide binding to LADH·NADH, excitation was at 320 nm and emission was observed at 415 nm.

Inner Filter Effect Corrections. For titrations with NADH as the variable ligand, increasing absorbance around 340 nm by NADH will diminish the intensity of light emitted by LADH in this region. Thus titration curves must be corrected for this "inner-filter" effect. So that this effect could be measured, titrations involving NADH around 10^{-5} M or above were continued well beyond apparent saturation, so that any further decrease in fluorescence was due to the inner-filter effect. This decrease was also monitored by titrating *N*-acetyltryptophanamide, present in amounts giving 294-nm absorbance equivalent to that of LADH, with the same concentrations of NADH used in actual experiments. The decrease in fluorescence by this method was identical with that obtained beyong the apparent end point of the initial uncorrected titration. The saturation point of the fluorescence quench was determined from the y intercept of a least-squares fit to a line drawn through the linear portion of the titration curve well beyond the end point.

Since the fluorescence quench should saturate beyong the end point, $F_{obsd} = F_{corrected}Q(N)$, where $Q(N)$ is a function of NADH concentration and corrects for NADH absorbance. Absorption of emitted light by NADH follows the Beer–Lambert law, and thus a plot of ln $Q(N)$ vs. [NADH] will be linear with a slope m. $F_{corrected}$ is then calculated as $F_{obsd}e^{m[NADH]}$. For titrations of LADH·NADH with *p*-(trifluoromethyl)benzamide, no inner filter effect correction was necessary since this ligand has no significant absorption near 320 or 415 nm.

Curve-Fitting Procedures. A nonlinear least-squares analysis for two phenomenological Adair constants, ψ_1 and ψ_2, and a different change in fluorescence upon saturation of *each* site of dimeric LADH was used to analyzed binding titrations. If F_1 represents the change in fluorescence upon saturation of the first-occupied site of the dimer and F_2 the change upon saturation of *both* sites, then the change in fluorescence for each point, ΔF_{obsd}, is compared to a calculated value ΔF_{calcd}, so that

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

where x is the free ligand concentration. The value of x can be related to x_0, the total concentration of ligand, by using the relationship

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

where E_0 is the enzyme concentration. This gives rise to a cubic equation in x. The appropriate root is selected and used with eq 1 to fit fluorescence as a function of total ligand x_0. The summed squared difference, $\sum_{\text{all points}}(\Delta F_{obsd} - \Delta F_{calcd})^2$, is refined until it reaches a minimum which then gives best-fit values of all parameters among ψ_1, ψ_2, F_1, and F_2, not arbitrarily held constant. Any combination of up to three parameters may be fixed for minimization at initial stages of data analysis.

A similar procedure was employed to fit slow exchange NMR binding data. Here the amplitude of the bound peak is measured as a function of total ligand concentration. When eq 1 is used, the bound intensity replaces F_{obsd} and since there is a linear relationship between NMR intensity and concentration of bound species, the parameter F_2 must equal $2F_1$. The values of F_1, ψ_1, and ψ_2 were allowed to vary until the best fit was obtained. The parameter F_1 is then proportional to enzyme site concentration (in arbitrary NMR intensity units), and ψ_1 and ψ_2 have their normal interpretation.

The Hill coefficient n_H was calculated directly from the best-fit Adair constants as $4/(\psi_1/\psi_2^{1/2} + 2)$ (Dahlquist, 1978).

Nonlinear Fluorescence Quench Corrections. NADH binding to LADH is known to result in a nonlinear quench of LADH fluorescence when excitation is near 290 nm and emission is in the 340-nm region. Due to the potential for a nonlinear fluorescence quench, a more complex treatment of binding data was demanded as outlined below:

(1) We first titrated LADH·alcohol with NADH, with E_0 = 0.4 μN, to estimate the tightness of NADH binding. Assuming a fluorescence quench that is linear with ligand binding, an apparent $K_d = (\psi_2)^{-1/2}$, where ψ_2 is the best-fit second Adair constant, is obtained. At pH 8.75, 25 °C, this was 0.15 μM (four experiments).

(2) The titration was then repeated at 28.9 μN LADH. Under these conditions, NADH binding should be stoichiometric since there was little indication of binding cooperatively in the initial titrations. This titration was used to fit for F_1 and F_2 by using the values for ψ_1 and ψ_2 derived from the earlier fits with 0.4 μN LADH as initial estimates. These values of F_1 and F_2 were held constant in the nonlinear least-squares procedures used at this point. (With these high concentrations of NADH, the data were first corrected for the inner-filter effect.) The values of F_1 and F_2 determined at high enzyme concentration were essentially independent of the ψ_2 value determined in step 1. Hence nonlinear effects in step 1 did not greatly change F_1 and F_2.

(3) The new best-fit values of ψ_1 and ψ_2 were now fixed, and data obtained with low concentrations of enzyme were reanalyzed by using these values.

(4) The new values of ψ_1 and ψ_2 were used to refine earlier estimates of F_1 and F_2 obtained in step 2.

Results

In Figure 1 the upper spectrum shows the ^{19}F NMR spectrum obtained when LADH, *p*-(trifluoromethyl)benzyl alcohol, and NADH are mixed in the proportion of 2.0:1.9:1.9 at pH 8.75 and 15 °C. A single resonance is observed 14.09 ppm downfield from TFA, shifted from the free alcohol resonance which comes at 13.44 ppm. This single resonance represents a fast-exchange average of the bound and free

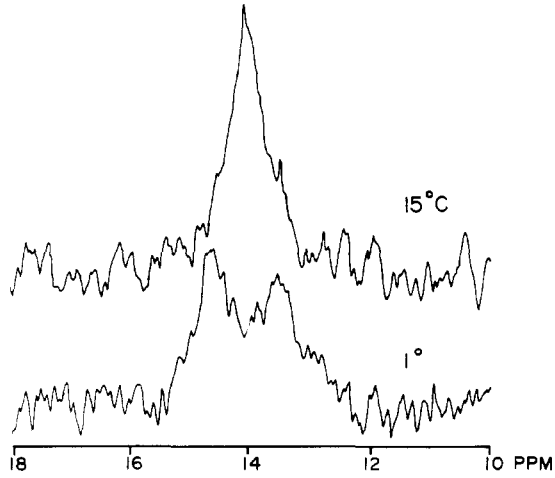

FIGURE 1: ^{19}F NMR observation of the temperature dependence of the exchange between two peaks observed when 0.198 mN LADH, 0.188 mM *p*-(trifluoromethyl)benzyl alcohol, and 0.212 mM NADH are incubated in pH 8.7 50 mM sodium pyrophosphate buffer. In the bottom spectrum, the two resonances are at 14.70 ppm, and the single peak appears at 14.09 ppm; 200 transients were accumulated for each spectrum, with a delay of 9 s between each transient.

contributions [see Anderson & Dahlquist (1980)]. However, a dramatic change in the spectrum of this sample is obtained when the temperature is lowered. When the temperature is decreased to 1 °C, two distinct peaks appear as shown in the lower spectrum of Figure 1. The downfield peak comes at 14.70 ppm while the upfield peak is at 13.50 ppm.

The different exchange properties of this complex at 1 and 15 °C have allowed us to assign the origin of the two peaks. Figure 2 shows spectra of LADH·NADH that were recorded with varying amounts of *p*-(trifluoromethyl)benzyl alcohol at pH 8.7 and 1 °C. The lowest spectrum (A) shows a single peak at 14.65 ppm, resulting when 0.53 mol of alcohol per mol of enzyme dimer is present. With the addition of more alcohol to a ratio of 1.06 per dimer in spectrum B, the single peak is still observable. With subsequent addition of alcohol in spectra C–E to 1.59, 2.12, and 3.45 per dimer, the upfield peak appears and grows in intensity. It narrows in width as alcohol is increased above the enzyme site concentration. Since only one upfield peak appears, this behavior suggests that it is in fast exchange with the free alcohol resonance or is the free alcohol resonance itself. The shift of the downfield peak is unaffected by the addition of superstoichiometric alcohol, suggesting that it represents an environment in slow chemical exchange with free alcohol.

For exchange of alcohol out of the bound environment, the only process affecting exchange is the unimolecular desorption rate. Thus if the downfield peak represents bound alcohol, it should have a concentration-independent exchange lifetime with the upfield peak. If the upfield peak represents free alcohol, it should have an exchange lifetime that depends on the free concentration of LADH. For data including that shown in Figure 2, the observed line width of the downfield peak is independent of the free enzyme (and thus free alcohol) concentration, while the line width of the upfield resonance is clearly concentration dependent. Thus the upfield resonance represents free alcohol, and the downfield resonance represents alcohol bound in a complex with LADH and NADH. No binary LADH·alcohol complex is present in Figure 2 to account for the upfield peak since free NADH is 2000-fold above the midpoint concentration (0.5 μM) derived from the NADH binding isotherm. Figure 3 shows the intensity of the downfield resonance plotted vs. total alcohol concentration, from the data of Figure 2. The equivalence point intersects the x axis at 0.32

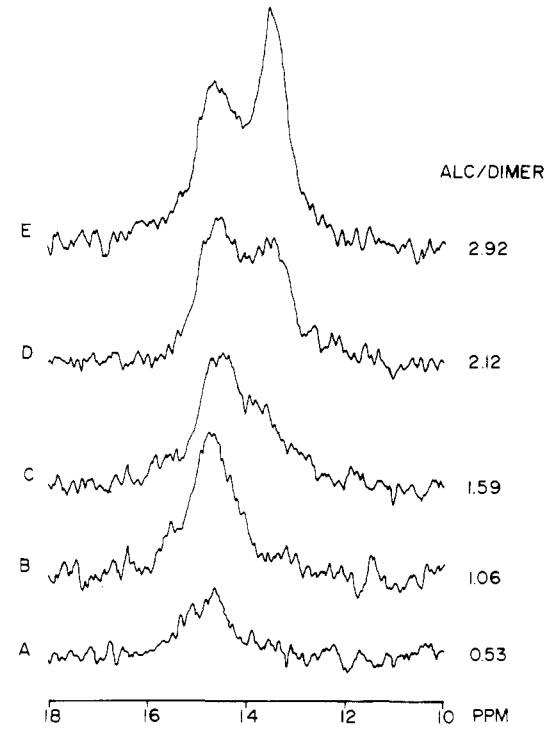

FIGURE 2: Titration of LADH plus NADH with *p*-(trifluoromethyl)benzyl alcohol observed by ^{19}F NMR. 0.29 mN LADH and 1.3 mM NADH were titrated at pH 8.7, 1 °C, with *p*-(trifluoromethyl)benzyl alcohol. The ratio of enzyme sites to NADH was constant for all spectra. In spectra A–E, the downfield peak occurred at 14.65, 14.64, 14.62, 14.62, and 14.68 ppm. The upfield peak, first discernible in spectrum C at about 13.6 ppm, occurs in spectra D and E at about 13.6 and 13.45 ppm. Spectra A and B were transformed from 600 and 720 transients, respectively, while spectra C–E resulted from 500 accumulated transients.

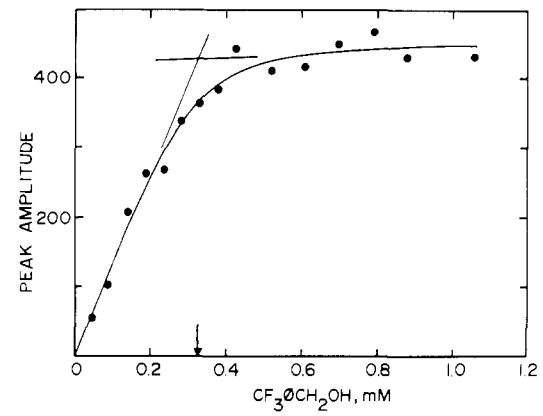

FIGURE 3: Plot of the intensity of the downfield *p*-(trifluoromethyl)benzyl alcohol resonance vs. total alcohol concentration. The data were taken from an experiment similar to that shown in Figure 2, except that an equal number of transients were collected for each spectrum. The data were fit to two Adair constants and a total amplitude, with $F_1 = 0.5F_2$. For the best-fit curve overlaying the data, $\psi_1 = 0.0705\ \mu M^{-1}$ and $\psi_2 = 0.00200\ \mu M^{-2}$. Also shown is the equivalence point of the titration, which intersects the x axis at 0.32 mM alcohol. The best-fit value of F_2 was 460.

mM alcohol, nearly equal to the site concentration of 0.29 mM. This suggests that the downfield peak accounts for all of the alcohol bound to LADH·NADH. The smooth line shown in the figure is the best-fit curve for binding at both enzyme sites. From the nonlinear least-squares best-fit parameters, the midpoint of the binding curve is 22 μM. Due to the nearly stoichiometric binding of alcohol, we are unable to determine ψ_1 (see Materials and Methods) and thus cannot determine a Hill coefficient for alcohol binding in this experiment.

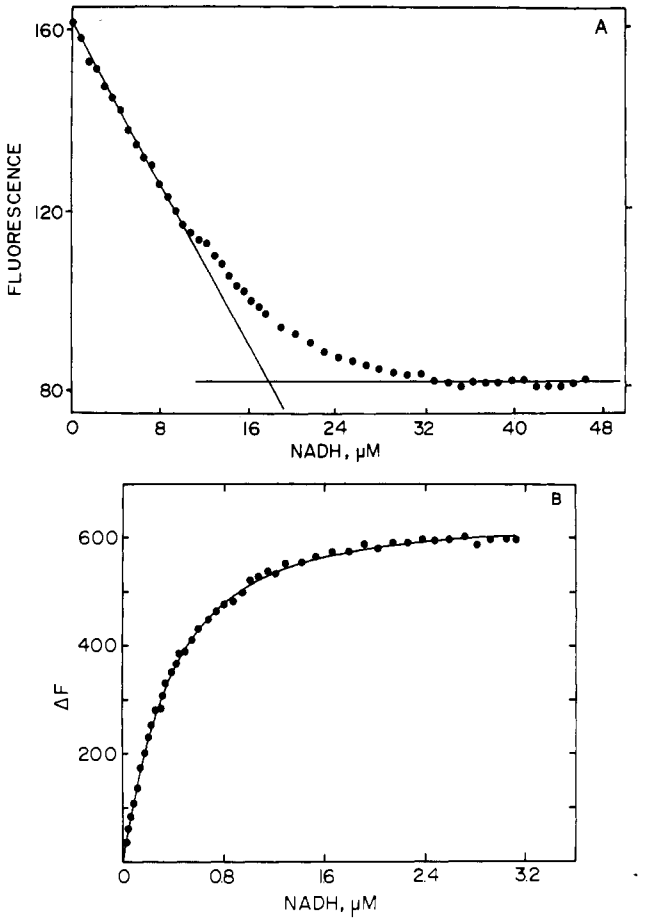

FIGURE 4: (A) Titration of concentrated LADH plus *p*-(trifluoromethyl)benzyl alcohol with NADH to establish a nonlinear quench of the LADH fluorescence. Titration of 28.9 μN LADH and 5 mM alcohol with NADH was observed by the quench in LADH fluorescence at 340 nm when excited at 290 nm. After correction for the inner-filter effect (see text), it was estimated that binding of the first NADH to the dimer resulted in 85% of the total observed fluorescence quench. The titration was in pH 8.75 50 mM sodium pyrophosphate buffer at 25 °C. (B) Titration of a low concentration of LADH plus *p*-(trifluoromethyl)benzyl alcohol with NADH to establish the binding isotherm. The quench of LADH fluorescence is observed at 340 nm when excited at 294 nm. 0.23 μN LADH and 5 mM alcohol were present. After correction for inner-filter effects, the best-fit binding isotherm was described by the Adair constants $\psi_1 = 4.39\ \mu M^{-1}$ and $\psi_2 = 3.58\ \mu M^{-2}$, resulting in a calculated Hill coefficient of 0.93. Titration was at pH 8.7 and 25 °C. *y*-axis numbers increase with increased quench of LADH fluorescence.

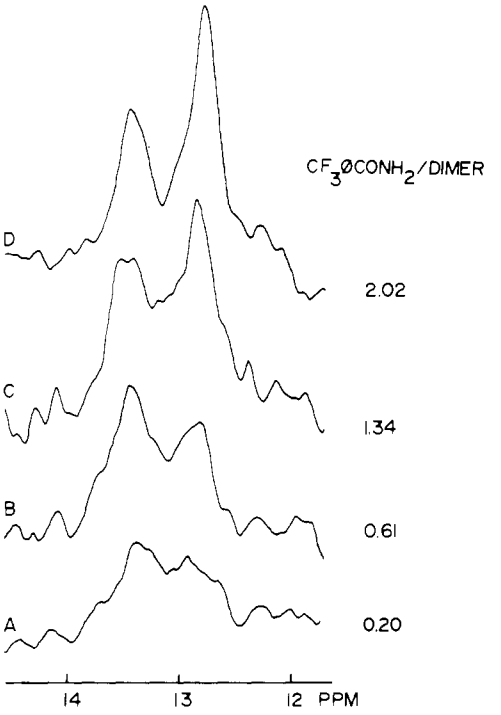

FIGURE 5: ^{19}F NMR titration of LADH·NADH with *p*-(trifluoromethyl)benzamide. Titration of 0.131 mN LADH plus 0.89 mM NADH with the amide at pH 8.7, 1 °C, is shown. The ratio of amide per dimer is shown at the right; the ratio of LADH to NADH was constant in all spectra. The downfield peak in spectra A–D occurs at about 13.4, 13.43, 13.50, and 13.55 ppm, respectively. The upfield peak occurs at about 13, 12.86, 12.83, and 12.77 ppm in spectra A–D. A variable number of scans were accumulated for these spectra, ranging from 13 500 in (A) to 300 in (D), with a delay of 6 s between transients.

To determine the binding isotherm of NADH to LADH·alcohol, we have exploited the quench of LADH fluorescence upon binding of NADH. Preliminary titrations, assuming a linear fluorescence quench, gave an apparent K_d of 15 μM, suggesting that binding of NADH to a 200-fold excess of LADH should be stoichiometric. Figure 4A shows the results of a titration by NADH of 28.9 μN LADH preincubated with 0.5 mM *p*-(trifluoromethyl)benzyl alcohol. The fluorescence appears to be linearly quenched for NADH additions up to a little beyond half-saturation. A decrease in fluorescence quench with further addition of NADH is observed. Iteration of steps 2–4 described under Materials and Methods results in a final estimate of F_1/F_2 from four titrations of 0.85 ± 0.05.

With this knowledge, the curves obtained with 0.4 μN LADH can be fit by using a fixed ratio of $F_1/F_2 = 0.85$. The final value of F_2 was adjusted to minimize the quantity $\sum_{\text{all points}}(\Delta F_{\text{obsd}} - \Delta F_{\text{calcd}})^2$ after initial estimates of F_2 were obtained from the *y* intercept of a plot of $1/\Delta F_{\text{obsd}}$ vs. 1/[NADH]. Figure 4B shows a binding curve fit by this procedure. For this curve, best-fit values of ψ_1 and ψ_2 of 4.4 μM^{-1} and 3.6 μM^{-2} were derived. From four experiments, an average Hill coefficient of 0.9 ± 0.1 was calculated from these best-fit values. The average concentration of free NADH at the midpoint of the titration, $(\psi_2)^{-1/2}$, was 0.46 ± 0.17 μM.

Use of p-(Trifluoromethyl)benzamide To Probe LADH Subunit Interactions. (A) ^{19}F NMR Titration of LADH·NADH with p-(Trifluoromethyl)benzamide. To approximate the properties of the active ternary complex LADH·NADH·aldehyde, which is not present in a large enough concentration to observe by ^{19}F NMR at equilibrium, we used the inactive aldehyde analogue *p*-(trifluoromethyl)benzamide. Benzamide and isobutyramide, as well as fatty acid amides, interact preferentially with LADH·NADH, as opposed to LADH·NAD, to form inactive ternary complexes (Sund & Theorell, 1963; Sigman, 1967). They also form binary complexes with LADH. In the case of isobutyramide, the affinity of NADH for LADH and isobutyramide at pH 7 is very strong ($K_d = 5 \times 10^{-9}$ M). Amides are competitive with aldehydes, but not alcohols [Winer & Theorell, 1960; see also Sund & Theorell (1963)], and may be useful for modeling the properties of the LADH·NADH·aldehyde complex.

We are able to observe the ^{19}F NMR spectrum of *p*-(trifluoromethyl)benzamide binary complexes with LADH at pH 8.75 and 1 °C (data not shown). Analysis of the chemical shift of the fast-exchange line between free and bound *p*-(trifluoromethyl)benzamide gave a best-fit K_d of 0.1 mM and bound shift of 0.54 ppm. This establishes a saturable complex formation of *p*-(trifluoromethyl)benzamide with LADH of moderate affinity.

We have also been able to use ^{19}F NMR to observe bound complexes of *p*-(trifluoromethyl)benzamide, NADH, and

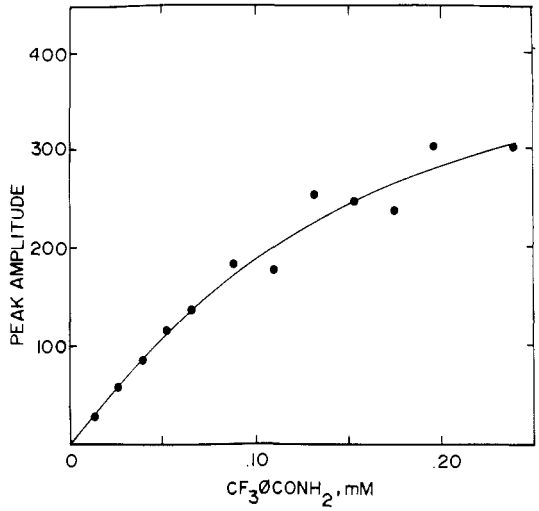

FIGURE 6: Plot of the intensity of the downfield *p*-(trifluoromethyl)benzamide peak vs. total amide concentration. The best-fit Adair constants were $\psi_1 = 0.426\ \mu M^{-1}$ and $\psi_2 = 3.25 \times 10^{-4}\ \mu M^{-2}$. A minimization of the saturated peak amplitude with F_1 held equal to $0.5F_2$ yielded a value of 430 for F_2. The best-fit curve is shown overlaying the data.

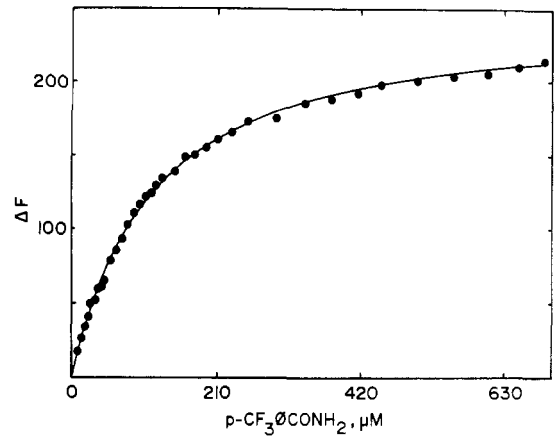

FIGURE 7: Titration of LADH·NADH with *p*-(trifluoromethyl)-benzamide by quench of the bound NADH fluorescence. Titration of 2.22 μN LADH and 108 μM NADH with amide at pH 8.7, 25 °C, was with excitation at 320 nm and emission at 415 nm. Best-fit parameters were $\psi_1 = 0.0171\ \mu M^{-1}$ and $\psi_2 = 7.66 \times 10^{-5}\ \mu M^{-2}$, giving a calculated Hill coefficient of 1.01. A linear fluorescence quench with binding was assumed. As the *y*-axis numbers increase, the quench of NADH fluorescence also increases.

LADH. Figure 5 shows the results when 0.031 mN LADH and 0.89 mM NADH are titrated with *p*-(trifluoromethyl)-benzamide at pH 8.7 and 1 °C. The lowest spectrum (A) results when 0.20 mol of the amide per mol of enzyme dimer is present. Peaks at 13.4 and around 13 ppm are evident. In spectrum B, both peaks are clearly present. In spectrum D, the added *p*-(trifluoromethyl)benzamide clearly begins to appear as intensity added to the upfield line. It has shifted to 12.77 ppm which is close to the shift of the resonance of free *p*-(trifluoromethyl)benzamide at 12.75 ppm. Thus the upfield line represents a fast-exchange average of bound and free amide or the free ligand itself, while the downfield resonance is in slow exchange with the free ligand resonance.

The exchange between the two resonances with substoichiometric amide exhibits a temperature dependence similar to that found for the LADH·NADH·alcohol complex (cf. Figure 1). By 15 °C both lines approach a fast-exchange average, with only a single intermediate resonance observable (data not shown). Treating the data as a binding isotherm in Figure 6 yields a midpoint for the binding curve of 55 ± 15 μM and a Hill coefficient of 0.9 ± 0.1. Thus *p*-(trifluoromethyl)benzamide binding to LADH·NADH appears effectively noncooperative, reinforcing the suggestion of only one type of binding site for this substrate analogue in its ternary complex with LADH and NADH.

(B) Fluorescence Titration of LADH·NADH with p-(Trifluoromethyl)benzamide. Since apparent nonhyperbolic binding of benzamide to LADH and NADH has been previously observed (Seydoux et al., 1974), we have also carefully examined *p*-(trifluoromethyl)benzamide binding to LADH·NADH by a method independent of ^{19}F NMR. No inner filter effect correction is necessary due to the lack of amide absorption at either the excitation wavelength of 320 nm or the emission wavelength of 415 nm. A typical binding isotherm is shown in Figure 7, with the best-fit solid line derived from the parameters $\psi_1 = (17 \pm 3) \times 10^{-3}\ \mu M^{-1}$ and $\psi_2 = (7.7 \pm 3) \times 10^{-5}\ \mu M^{-2}$. These give a Hill coefficient of 1.01 and for eight experiments an average Hill coefficient of 1.04 ± 0.05. The results give no indication of site–site interaction in the LADH·NADH·*p*-(trifluoromethyl)benzamide complex.

Relative Stabilities of LADH·NAD·Alcohol and LADH·NADH·Alcohol Ternary Complexes. We can now determine

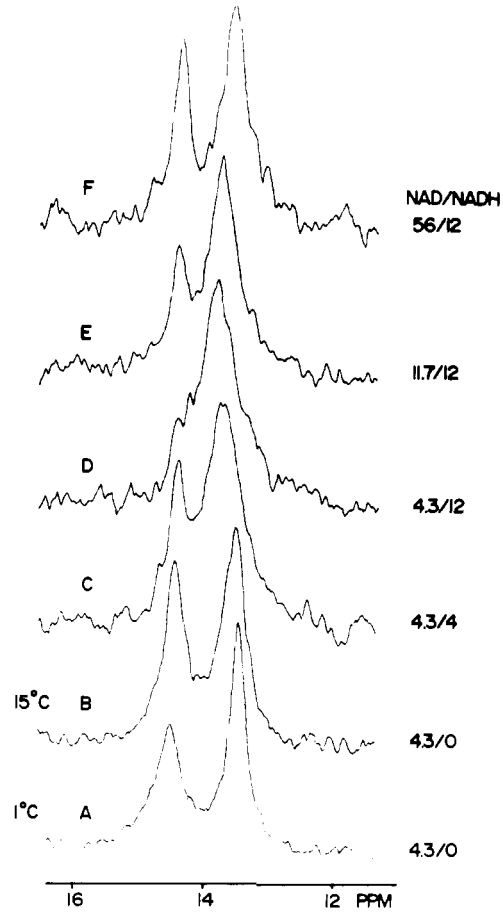

FIGURE 8: Determination of the relative affinity of *p*-(trifluoromethyl)benzyl alcohol for LADH·NAD and LADH·NADH by ^{19}F NMR. The spectrum due to 0.328 mN LADH preincubated with 0.64 mM alcohol and 0.698 mM NAD at pH 8.7 is shown in (A) at 1 °C and in (B) at 15 °C. All spectra subsequent to (B) are at 15 °C, with the ratio of NAD/NADH shown at the right. The ratio of alcohol to LADH remained constant throughout. All peak positions are given in the text. Approximately 100 transients are accumulated for each spectrum with a delay of 8 s between transients. All apparent peak positions were determined from best-fit Lorentzian lines.

the relative affinity of *p*-(trifluoromethyl)benzyl alcohol between its active ternary complex with LADH·NAD and its

abortive ternary complex with LADH·NADH due to the differing exchange properties with free alcohol in these two complexes. In Figure 8, spectra A and B show the exchange properties of the LADH·NAD·alcohol complex with free alcohol. At both 1 and 15 °C, the resonance of *p*-(trifluoromethyl)benzyl alcohol bound in its ternary complex with LADH·NAD (14.48 ppm) is in slow exchange with the resonance of free alcohol at 13.44 ppm. Under conditions that will produce both ternary complexes, a predominance of the LADH·NAD·alcohol peak at 15 °C will be reflected by the presence of a slow-exchange peak at about 14.45 ppm, while a predominance of the LADH·NADH·alcohol complex will be reflected in the presence of a broad intermediate to fast-exchange peak somewhere in position between the two peaks observed at 1 °C, i.e., between 14.65 ppm and 13.65 ppm. The exact position will depend on the relative ligand population in each environment.

Spectrum C results when equal amounts of NAD and NADH are allowed to compete for LADH·alcohol at 15 °C. The upfield resonance appears at 14.38 ppm, suggesting it is due to the LADH·NAD·alcohol complex. A larger, broader resonance has appeared around 13.72 ppm. Its width suggests it might be due to intermediate to fast chemical exchange (as in Figure 1). This possibility is supported by addition of more NADH in spectrum D. Under these conditions 12.0 NADH and 4.3 NAD are present for every 2 LADH sites. The resonance at 14.38 ppm has diminished considerably in intensity. The main resonance is now at 13.81 ppm, having moved downfield from 13.7 ppm. Spectra in E and F show that the 14.38-ppm resonance and appearance of the 13.8-ppm resonance can be reversed with addition of NAD. In spectrum F a large excess of NAD is present, with the resulting spectrum appearing essentially identical with that of spectrum B (when no NADH was present).

If one assumes that the exchange properties of each ternary complex in LADH(NAD·alc)(NADH·alc) are predictable from those of the separate homo dimers, the above spectra can be interpreted in a straightforward fashion. The appearance of the broad line in spectra C and D of Figure 8 is due to the LADH·NADH·alcohol complex, which is favored more in spectrum D with the addition of excess NADH. This would diminish the resonance due to LADH·NAD·alcohol; thus the 14.38-ppm resonance is barely detectable in spectrum D. The averaged resonance in spectrum D has moved downfield, which would be expected with the formation of more LADH·NADH·alcohol. It can never move to 14.1 ppm, the theoretical position half-way between the two peaks observed at 1 °C, because its position is weighted by the presence of 2.3 free alcohols per dimer, which rapidly exchange with all environments but LADH·NAD·alcohol at 15 °C.

The predominance of the broad resonance at 13.8 ppm in spectrum D and the reversal in spectrum F to the original spectrum of B suggest that *p*-(trifluoromethyl)benzyl alcohol has similar affinity for LADH·NAD and LADH·NADH.

Discussion

In a philosophical sense one can never show site equivalence, only that a given technique does not distinguish among sites within its sensitivity. For this reason we have employed techniques which allow the highest degree of sensitivity to site equivalence short of X-ray crystallography. Using fluorine nuclear magnetic resonance, we can determine if two sites (a) have distinguishable chemical environments as determined by the exquisite chemical shift sensitivity of the fluorine nucleus, (b) have distinguishable kinetics of exchange of bound and free ligand, and (c) have distinguishable equilibrium thermodynamics of ligand interaction. The ^{19}F NMR equilibrium measurements have been supplemented with measurements of ligand binding using enzyme or coenzyme fluorescence quenching.

To probe the environmental equivalence of the two active sites of LADH, we have looked carefully at the abortive complex of LADH·NADH with the substrate *p*-(trifluoromethyl)benzyl alcohol, the formation of which has been suggested to be a trigger for reaction at adjacent subunits (Baici & Luisi, 1977), and the complex of LADH·NADH with the aldehyde analogue *p*-(trifluoromethyl)benzamide. For both complexes, the NMR spectra show profound changes in the ligand resonances in the presence of LADH. In each case we are able to observe two separate resonances in the slow-exchange limit. The downfield peak represents alcohol or amide adsorbed in a ternary complex with NADH, while the upfield peak represents free alcohol or amide which is broadened extensively by exchange with the bound ligand.

Titrations of the intensity of the downfield resonance with *p*-(trifluoromethyl)benzyl alcohol or *p*-(trifluoromethyl)-benzamide show that the downfield peak represents only one bound environment for both sites of the dimer. Since the equivalence point of these titrations equals the total enzyme site concentration, by ^{19}F chemical shift criteria only one homogeneous environment is detected.

There appears to be essentially only one alcohol exchange rate out of its ternary complex with LADH·NADH. If there were two rates, the observed downfield peak would appear as the superposition of a broad component, exchanging at an intermediate rate with free alcohol, and a sharper peak, exchanging slowly with free alcohol. The observed resonance shows no such compound shape and is well described as a single Lorentzian line. This is also true for the exchange of *p*-(trifluoromethyl)benzamide out of its ternary complex with enzyme and NADH.

One measure of cooperativity between enzyme subunits is derived from the Hill coefficient. In light of the qualitative report of cooperative binding of benzamide to horse LADH in the presence of NADH (Seydoux et al., 1974), we determined the binding isotherms of its trifluoromethyl analogue in the same complex with saturating levels of NADH and carefully determined the Hill coefficient by two independent methods. The Hill coefficient of the ^{19}F NMR derived binding isotherm determined at high protein concentration (0.92) agrees reasonably well with the same parameter derived from fluorescence quench measurements determined at low protein concentrations (1.01). For further correlation of these results, the Hill coefficient derived for the corresponding alcohol in the same complex is 0.89. There is no cooperativity seen between the dimers with this amide or the corresponding alcohol in the ternary complex with LADH and NADH. Thus we find no evidence for site heterogeneity with respect to the chemical environment as detected by ^{19}F chemical shift, observed desorption rate, or site cooperativity of alcohol or benzamide with their respective ternary complexes.

The observed rapid equilibrium exchange rate for alcohol out of its abortive ternary complex has other mechanistic implications as well. The rate of exchange must be greater than 2π(chemical shift upon binding in Hz) in order to give a fast exchange averaged measurement at room temperature. For the alcohol in ternary complexes with NADH at 15 °C, this will be about 770 s^{-1}. This eliminates the use of this ternary complex as a kinetic trap in the LADH reaction mechanism. Exchange of alcohol in and out of this complex cannot limit the slow step of the observed biphasic transient

kinetics, which has a rate of 4 s^{-1} for the oxidation of *p*-(trifluoromethyl)benzaldehyde (Anderson & Dahlquist, 1980). However, as suggested by the similar affinities of LADH·NADH and LADH·NAD for alcohol, the LADH·NADH complex can effectively compete with LADH·NAD for free alcohol and may thus act as a thermodynamic sink for enzyme sites, removing them from catalysis. In fact, when the initial velocity of LADH catalysis of the reaction between NAD and *p*-(trifluoromethyl)benzyl alcohol is observed as a function of alcohol concentration under the same conditions (data not shown), marked substrate inhibition is seen for *p*-(trifluoromethyl)benzyl alcohol concentrations above 1 mM.

This type of inhibition has been thought to correlate with changes in the transient kinetics of benzyl alcohol oxidation when observed by stopped-flow experiments (Baici & Luisi, 1977). At high alcohol concentrations they suggest that some enzyme sites are bound with NADH·alcohol. Our results indicate that this may well be happening. In order to propose a half-site reactive mechanism when stopped-flow burst amplitudes are more than 50% of the total amplitude, they suggest that the mixed hetero dimer LADH(NADH·alc)(NAD·alc) is rapidly formed and is subsequently reactive. Formation of the LADH·NADH·alcohol complex on one site causes reaction at the other site to give LADH·NADH·aldehyde, followed by aldehyde desorption.

We have seen no evidence for cooperativity in binding of *p*-(trifluoromethyl)benzyl alcohol in complexes with LADH and NAD (Anderson & Dahlquist, 1980; Anderson, 1980) or LADH and NADH. Thus when NAD, NADH, and alcohol are mixed with high concentrations of LADH, mixed hetero dimers of LADH(NAD·alc)(NADH·alc) should occur. Aromatic aldehydes are thought to have a low affinity for the LADH·NADH complex (Weidig et al., 1977; Anderson, 1980). If the above hypothesis of Baici & Luisi (1977) is correct and mixed hetero dimers are reactive, free aldehyde should be produced, resulting in a resonance around 12.46 ppm (Anderson & Dahlquist, 1980). This is not seen. Results of experiments with LADH, H_2NADH, and *p*-(trifluoromethyl)benzaldehyde (Anderson, 1980) suggest that any LADH·NADH·aldehyde present might give a single resonance at 13.11 ppm. This is also not seen, and all of the peak intensity appears to reside in resonances assigned to complexes of alcohol with LADH and NADH or LADH and NAD. Thus we find no evidence for any activity of the mixed hetero dimer at equilibrium, and our data do not support the aforementioned use of nonequivalent sites in explaining biphasic transient kinetics of this enzyme.

A thorough examination of a number of different LADH ternary complexes, some of which have been suggested to exhibit nonequivalent sites, has demonstrated only evidence for site homogeneity. We see absolutely no evidence for site heterogeneity in this system in bound complexes involving NADH or NAD. Since the ^{19}F probe is environmentally quite sensitive, any differences between enzyme sites must be quite subtle, if they exist. We see clear homogeneity in the exchange behavior of the two sites, suggesting that any differences that are not detectable (i.e., a difference of less than a factor of 2 in the exchange rates) are likely to be inconsequential for the catalytic mechanism of this enzyme.

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