Carbon-13 Nuclear Magnetic Resonance Studies on Liver Alcohol Dehydrogenase Specifically Alkylated with Bromo[1-^{13}C]acetate†

Raja G. Khalifah* and William McIver Sutherland

ABSTRACT: The carboxymethylation of horse liver alcohol dehydrogenase with bromoacetate was studied at pH 7.4 and compared to previous alkylations with iodoacetate and iodoacetamide. Stimulation of the alkylation rate was observed with imidazole at concentrations below 5 mM and protection at higher concentrations, while adenosine 5′-monophosphate, adenosine 5′-diphosphoribose (ADP-ribose), and decanoate afforded protection. The similarities with iodoacetate alkylation suggest a specific S-carboxymethylation of Cys-46, the ligand of the "catalytic" zinc. Use of 90% bromo[1-^{13}C]acetate to alkylate LADH thus introduces an enriched carboxylate ^{13}C nuclear magnetic resonance probe into the active site whose signal proved to be readily detectable at 180.6 ppm from Me_4Si. This chemical shift is 2 ppm downfield from its position in small model *S*-carboxymethyl compounds. The origin of this difference is unlikely to be due to the coordination of the zinc at the thioether sulfur of CmCys-46, although this coordination cannot be excluded. Characteristic shifts in the carboxylate resonance occur in complexes of the enzyme with the coenzymes NAD^+ (181.8 ppm) and NADH (179.6) and their fragment ADP-ribose (179.4), as well as in ternary complexes with inhibitors such as 4-iodopyrazole (177.8) and the substrate analogue trifluoroethanol (180.2). These results are interpreted in terms of a different binding mode for NAD^+ compared to that of NADH and ADP-ribose, but the addition of the substrate analogue apparently induces NAD^+ to bind in the proper conformation for catalysis. No pH perturbations have been detected in CmLADH and its complexes.

The structure and function of the equine liver alcohol dehydrogenase (EC 1.1.1.1), or LADH,[1] have received intensive study in recent years (Thurman et al., 1973, 1977, 1978), culminating in the high-resolution crystal structure determination of the enzyme and its complexes with coenzymes and inhibitors (Bränden et al., 1975; Plapp et al., 1978). Nevertheless, important mechanistic aspects of this metalloenzyme remain to be elucidated. Among these one can list the role of the "catalytic" and "noncatalytic" zinc atoms (Drum et al., 1967), the nature of the proton-linked active-site group(s) (Dunn, 1975; Shore et al., 1974), and the possible existence of negative cooperativity (Bernhard et al., 1970; Hadron et al., 1975; Baici & Luisi, 1977; Weidig et al., 1977). In addition, conformational changes associated with the binding of coenzymes (Bränden, 1965; Coates et al., 1977) or analogues (Samama et al., 1977) have been observed, but their mechanistic implications remain obscure.

Previous physical studies of LADH in solution have tended to emphasize properties of either coenzymes and ligands or spectroscopic and paramagnetic properties associated with metals that replace the zinc atoms such as Co^{2+} (Drum & Vallee, 1970; Ulmer & Vallee, 1971; Sytkowski & Vallee, 1978; Drott, 1978; Mildvan & Weiner, 1969; Sloan et al., 1975a,b; Young & Mildvan, 1978; Drott et al., 1978). We have sought an independent approach that emphasizes the NMR observation of tightly or covalently bound groups in the active site of this 80 000 dalton (dimeric) enzyme. We report here the successful introduction of a ^{13}C NMR "probe" into the active site, making use of a selective alkylation with bromo[1-^{13}C]acetate. Since previous alkylations on this enzyme have been done with either iodoacetate or iodoacetamide, we also compare the carboxymethylation characteristics of these reagents. Our ^{13}C NMR results to date indicate that this approach may be very promising for elucidating interactions in the active site. It is relevant to point out that the carboxymethylated enzyme is catalytically active (Reynolds & McKinley-McKee, 1970, 1975; Hardman, 1976), and its crystal structure has been determined to 0.45-nm resolution (Zeppezauer et al., 1975). A preliminary account relevant to these results has appeared (Khalifah, 1978).

Experimental Procedures

Enzymes. Horse liver alcohol dehydrogenase was purchased from Boehringer Mannheim (lots 1515242 and 1037243) as a suspension in phosphate buffer containing 10% ethanol. This enzyme has been shown to be more than 95% in EE subunits (Pietruszko et al., 1966). Enzyme concentration was calculated from the absorbance at 280 nm, assuming a 1 mg/mL extinction coefficient of 0.455 (Bonnichsen, 1950).

Enzyme Assays. The activity was assayed towards ethanol in pH 8.8 pyrophosphate buffers by following the increase in NADH absorbance at 340 nm according to the procedure of Drum et al. (1969).

Carboxymethylation Kinetics. The kinetics of reaction of LADH with $BrAc^-$ was followed by activity assays. Predialyzed enzyme samples were incubated at 25 °C in the dark with known concentrations of $BrAc^-$ that were sufficiently in excess to provide pseudo-first-order conditions. All reactions were carried out in 40 mM phosphate buffer of pH 7.40. At intervals, small aliquots of the reaction mixture were taken directly into standard assay solutions and were assayed immediately. Unreacted $BrAc^-$ was thus not quenched, except by the dilution, since the addition of dithiothreitol or mercaptoethanol was found to inhibit the enzyme. The rate constants were obtained by weighted least-squares analysis of semilogarithmic plots of $\ln (U_t - U_\infty)$ vs. time (the data usually extended to 3 half-lives), constant absolute errors in U being assumed.

NMR measurements were made on LADH carboxymethylated as follows. Stock LADH suspensions were dialyzed

† From the Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901. *Received September 25, 1978.* This research was supported in part by a U.S. Public Health research grant and by funds from the University of Virginia.

[1] Abbreviations used: LADH, horse liver alcohol dehydrogenase (EE isozyme); NMR, nuclear magnetic resonance; CmCys, *S*-carboxymethyl-L-cysteine; $BrAc^-$, bromoacetate; IAc^-, iodoacetate; $IAcNH_2$, iodoacetamide; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ADP-ribose, adenosine 5′-diphosphoribose; CSA, chemical shift anisotropy; GSH, reduced glutathione.

0006-2960/79/0418-0391$01.00/0

overnight against a large excess of cold 40 mM phosphate buffer of pH 7.40. Alkylations were then carried out in the same buffer but at room temperature in the absence of light. Typically, reaction mixtures contained 0.12 mM (in subunits) LADH and 1.2–1.4 mM bromo[1-^{13}C]acetate, along with 9–10 mM imidazole added to enhance the selectivity of the active-site reaction (see Results). The progress of alkylation was carefully monitored by activity assays, the half-time being usually about 25–30 min. The alkylation was terminated after the residual activity had decreased to 5–10% only. Excess reagent and side products were immediately removed by gel filtration on a Sephadex G-25 column operated at 4 °C. Pooled enzyme fractions were then concentrated by vacuum dialysis and were dialyzed against buffer if needed.

S-Carboxymethylation of Model Compounds. The *S*-carboxymethyl derivatives of reduced glutathione, *N*-acetyl-L-cysteine, and *N*-acetyl-DL-methionine were prepared by reaction with 90% bromo[1-^{13}C]acetate (cf. Gurd, 1972). In each case, about 0.5–1.0 μL of $BrAc^-$ was treated with a two–fivefold molar excess of the sulfhydryl compounds in a volume of 1 mL. GSH and *N*-Ac-L-Cys were reacted for several hours at room temperature at pH 7.4, while *N*-Ac-DL-Met was reacted overnight at a pH of 2.2. Isolation and purification of products were not done since the ^{13}C NMR signals of the enriched carboxylate could be easily observed and assigned under conditions where the natural abundance carbonyl carbons were negligble.

NMR Measurements. ^{13}C NMR spectra were obtained on a JEOL PFT-100/EC 100 Fourier transform spectrometer operating at a ^{13}C resonance frequency of 25.15 MHz. All spectra were obtained under full proton noise-decoupling conditions at 25 °C using a spectral width of 5000 Hz. Samples of about 0.8 mL were contained in 10-mm NMR tubes equipped with Teflon vortex plugs. Chemical shifts were measured relative to internal dioxane (1–2 μL) and were converted to the Me_4Si scale using 67.40 ppm as the dioxane shift. Recycle times were 0.9 s (FID's of 8 K), and the flip angle was chosen (Ernst and Anderson, 1966) to be about 35° based on the expected relaxation properties of carboxymethyl carboxyls (cf. Jeffers et al., 1978). Besides exponential multiplication, signal-to-noise was improved by zero-filling. All samples were made 10–15% in D_2O in order to provide an internal lock signal for the spectrometer. Enzyme concentrations were about 0.6–0.9 mM in subunits and samples were buffered to maintain pH control (cf. Khalifah et al., 1977).

Chemicals. The coenzymes NAD^+ and NADH were Grade III Sigma products. NADH was obtained in preweighed vials of 2 mg each and was dissolved just before use directly in enzyme samples of pH 8.7. NAD^+ was freshly dissolved before NMR use and was kept frozen for assay use. It was also purified in some control NMR experiments by passage on Sephadex G-10 at 4 °C. Sephadex G-10 and G-25 were obtained from Pharmacia. The 90% bromo[1-^{13}C]acetic acid was purchased from KOR Isotopes and its isotopic purity was previously confirmed (Khalifah et al., 1977). Unenriched bromoacetic acid and 2,2,2-trifluoroethanol (>99% purity) were obtained from Aldrich Chemicals. Imidazole, from Sigma, was recrystallized twice from acetone–petroleum ether before use. 5′-AMP was from P-L Biochemicals. 4-Iodopyrazole, decanoic (capric) acid, dithiothreitol, reduced glutathione, *N*-Ac-L-Cys, *N*-Ac-DL-Met, and adenosine 5′-diphosphoribose were all purchased from Sigma.

^{13}C NMR Line Width Determinations. The *intrinsic* line widths of observed resonances of CmLADH were estimated by subtracting the observed line width of the internal dioxane marker in each sample from the protein resonance. The implicit assumptions involved are that W_{obsd} contains contributions from field inhomogeneity (W_{ins}), from digital filtering due to exponential multiplication of the free induction decay signals (W_{exp}), and from the intrinsic line width (W_0) that arises from various relaxation mechanisms such as dipolar interactions with protons, chemical shift anisotropy (CSA), etc. Thus the *difference* in observed line widths between a protein resonance and the dioxane standard will not contain contributions except from the intrinsic factors:

$$W_{obsd}(\text{prot}) - W_{obsd}(\text{diox}) = W_0(\text{prot}) - W_0(\text{diox})$$

In our estimates we have set this difference equal to W_0(prot) only, i.e., we have ignored the dioxane intrinsic line width. This can be justified to a first approximation since the W_0 for dioxane can be theoretically estimated to be less than 0.5 Hz if the rotational correlation time of dioxane is typical of small molecules, i.e., less than about 4×10^{-11} s rad^{-1}. Theoretical expressions for calculation of dipolar and CSA contributions to the line widths of carboxylate carbons and methylene carbons are available in the literature (cf. Farrar & Becker, 1971; Jeffers et al., 1978).

Amino Acid Analysis. Protein hydrolysates were analyzed on a Glenco AS 100 system equipped with Durrum DC-1A resin and using single-column methodology. Samples were evacuated and hydrolyzed in 6 N HCl for 24 h at 110 °C before analysis.

Table I: Comparison of LADH Alkylations with Haloacetates

quantity	reagent $BrAc^-$[a]	reagent IAc^-[b]
k_o (min^{-1})	0.12	0.078
K_R (mM)	8.4	4.5
k_o/K_R (M^{-1} min^{-1})	13.4[c]	17.3[c]
K_i (μM) for AMP protection	41	32
K_i (μM) for ADP-ribose protection	8.8	10.5
K_i (mM) for imidazole stimulation	~1[d]	0.86

[a] This work, using 40 mM phosphate buffers of pH 7.40 at 25 °C. [b] Results of Reynolds & McKinley-McKee (1969) and Reynolds et al. (1970) obtained in 40 mM phosphate buffers of pH 7.40 at 23.5 °C. [c] For comparison, the second-order inactivation rate constant for iodoacetamide is about 1.3 M^{-1} min^{-1}, as estimated from Figure 2 of Evans & Rabin (1968). [d] A protective effect is also exhibited by imidazole at higher concentrations (see text).

Results

Carboxymethylation of LADH with $BrAc^-$. As with iodoacetate (Li & Vallee, 1963), millimolar concentrations of bromoacetate inactivate LADH in a time-dependent reaction typical of covalent modifications. The inactivation appears, however, to leave intrinsic residual activity in the modified enzyme at the 1% level by our assay, as judged at very long reaction times (~10 half-times). The kinetics of inactivation is strictly first order in time, yielding linear semilogarithmic plots of ln ($U_t - U_\infty$) vs. time up to at least 3 half-lives (cf. Figure 2). The apparent first-order rate constant k deduced from such plots shows a hyperbolic ("Michaelis–Menten") dependence on the concentration of $BrAc^-$. This behavior is typical of a situation where inactivation is dependent on *reversible* binding of the reagent (cf. Reynolds & McKinley-McKee, 1969). Analysis of the data by a suitable linear plot (Figure 1) yields the maximal rate constant k_o at saturating $BrAc^-$ and the apparent dissociation constant K_R for the reversible binding of the $BrAc^-$ reagent (Table I).

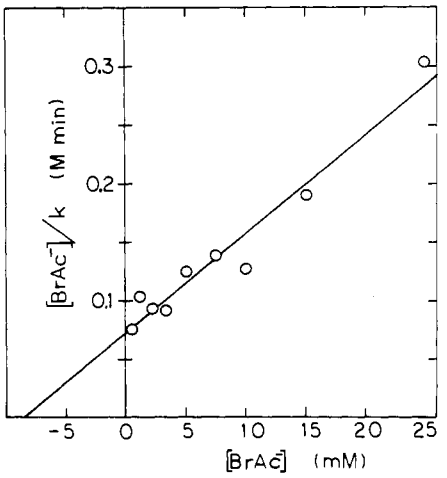

FIGURE 1: Linear plot of the dependence of the apparent rate constant for LADH inactivation by $BrAc^-$ on the reagent concentration in 40 mM phosphate buffer of pH 7.4 at 25 °C. Plot is a linear transform of a simple hyperbolic ("Michaelis–Menten") dependence of k on [$BrAc^-$]. The reciprocal of the slope is the apparent dissociation constant K_R for $BrAc^-$ *reversible* binding, and the ratio of the y intercept to the slope is the maximal (saturating) rate constant k_o for *covalent* inactivation. The line represents a linear least-squares fit to the data.

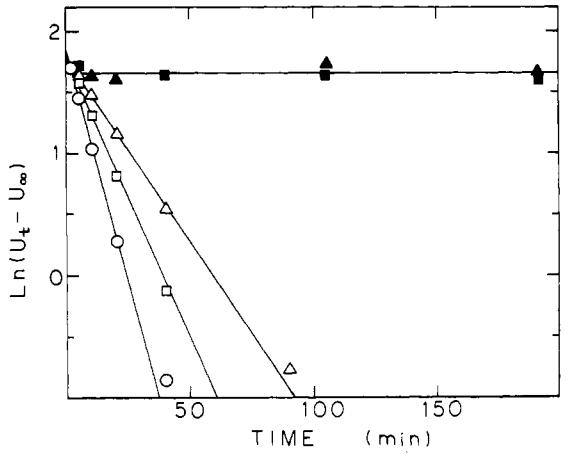

FIGURE 2: Semilogarithmic plots for the rate of reaction of 10 mM $BrAc^-$ with 0.9 μM LADH at pH 7.40 in phosphate buffer containing inhibitors as follows: (○) control; (□) 52 μM AMP; (■) 0.26 mM AMP; (△) 30 μM ADP-ribose; (▲) 0.15 mM ADP-ribose. U is the specific activity towards ethanol.

Inhibitor Protection against $BrAc^-$ Inactivation. The LADH inactivation with IAc^- is known to be inhibited by coenzymes (Li & Vallee, 1963) and other ligands or inhibitors of the enzymatic activity (Evans & Rabin, 1968; Reynolds & McKinley-McKee, 1969; Reynolds et al., 1970). A similar situation occurs, as expected, with $BrAc^-$. Thus AMP, ADP-ribose, and decanoate (not shown) afford almost complete protection at saturating concentrations and partial protection at lower concentrations (Figure 2). The latter permits the calculation of the dissociation constants for the protecting inhibitors if it is assumed that competitive reversible binding occurs between $BrAc^-$ and the inhibitors. The applicable relation is

$$k^I/k = (1 + [R]/K_R)/(1 + [R]/K_R + [I]/K_i) \quad (1)$$

where k^I and k are the apparent rate constants for inactivation in the presence and absence, respectively, of the protecting inhibitors I, while [R] is the $BrAc^-$ concentration. It is assumed that [I] and [R] are in large excess over the enzyme. The data of Figure 2 yield by this analysis the K_i values given in Table I, where they are compared with previous results using the IAc^- modification.

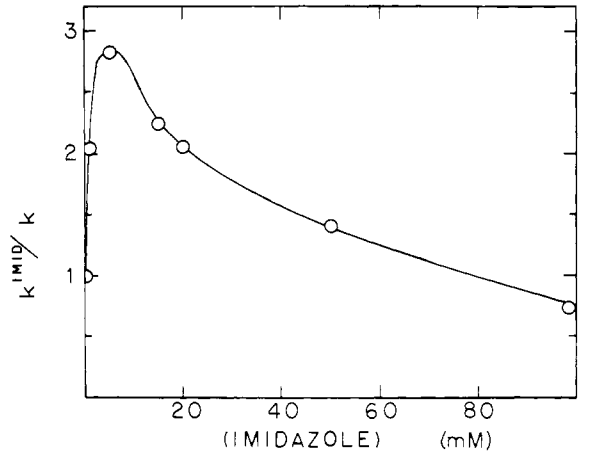

FIGURE 3: Effect of imidazole on the rate of inactivation of 1.1 μM LADH with 0.5 mM $BrAc^-$ in 40 mM phosphate buffer of pH 7.4 at 25 °C. The ordinate gives the ratio of the apparent first-order rate constant in the presence of the indicated concentrations of imidazole to that in the absence of imidazole.

Table II: ^{13}C Chemical Shifts of Carboxylates in Model Compounds

compound	pH	chemical shift[a]
$BrCH_2C^*OO^-$	7.3	175.6
S-($CH_2C^*OO^-$)-GSH	6.0–10.6	178.4
S-($CH_2C^*OO^-$)-N-Ac-L-Cys	7.4	178.6
S-(CH_2C^*OOH)-L-Cys	<0.5[b]	175.0
S-($CH_2C^*OO^-$)-N-Ac-D,L-Met	6.9	169.0
N^τ- or N^π-($CH_2C^*OO^-$)-L-His	4.0	173.0 ± 0.2[c]
N^τ- or N^π-($CH_2C^*OO^-$)-L-His	9.0	176.1 ± 0.2[c]

[a] In parts per million downfield from Me_4Si assuming internal dioxane at 67.40 ppm. [b] In 2 M HNO_3. [c] Taken from Khalifah et al. (1977). The two values refer to conditions where the imidazole ring is protonated or deprotonated.

Imidazole Stimulation and Protection. In contrast with other inhibitors, imidazole, which binds at the catalytic zinc (Boiwe & Bränden, 1977), enhances the active-site covalent modification with IAc^- and $IAcNH_2$ (Evans & Rabin, 1968). A parallel situation occurs with $BrAc^-$, as shown in Figure 3. However, higher concentrations of imidazole reverse this trend and cause retardation. The midpoint for the stimulation effect is about 1 mM (Table I). We have utilized this effect to enhance the selectivity of the LADH carboxymethylation when preparing the CmLADH for NMR study (see above).

^{13}C NMR Shifts of S-Carboxymethyl Model Compounds. We have measured the carboxyl chemical shift of $BrAc^-$ and the S-[^{13}C]carboxymethyl derivatives of GSH, N-Ac-L-Cys, and N-Ac-DL-Met in order to help interpret the spectra of CmLADH. The shift data are given in Table II, along with previously obtained results on the carboxymethyl carboxyl of N^τ- and N^π-carboxymethyl-L-histidine (Khalifah et al., 1977).

^{13}C NMR of Enriched CmLADH. The ^{13}C NMR spectrum of enriched CmLADH, prepared as described in Experimental Procedures, is shown in Figure 4A. In the carbonyl region (165–185 ppm) one can observe the natural abundance envelope of all the carbonyl, carboxyl, and carboxamido carbons of the enzyme centered close to 173 ppm. However, a large and relatively sharp resonance (~5 Hz intrinsic line width) occurs downfield of this envelope at 180.6 ppm in a region normally devoid of strong signals. This resonance cannot be removed by extensive dialysis, and its chemical shift implicates it as being due to an S-[^{13}C]carboxymethylcysteinyl carbon (cf. Table II). Since the chemical shift is unique and since

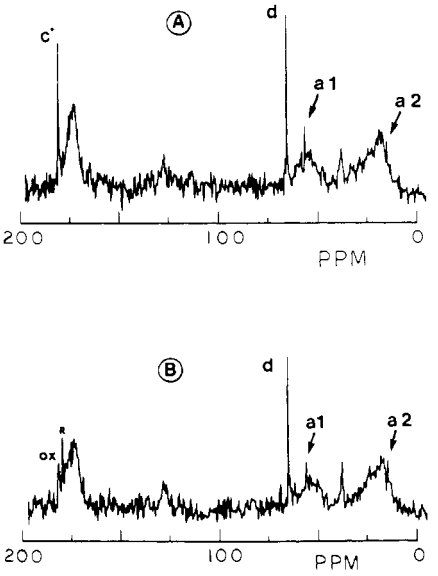

FIGURE 4: 25.15-MHz ^{13}C NMR spectrum of 0.9 mM LADH carboxymethylated with 90% [1-^{13}C]bromoacetate in the absence (A) and presence (B) of 3.1 mM NAD^+. Spectra were the result of 63 600 transients obtained using 8K frequency domain data points, 5000-Hz spectral width, 0.9-s recycle time, 30° flip angle, and data smoothing equivalent to 3.5-Hz line broadening. Buffer was 40 mM phosphate of pH 7.40 at 25 °C. (A) The peak marked C* is the resonance of the enriched carboxylate, d is the internal dioxane marker, and peaks a-1 and a-2 are resonances of C-1 and C-2 of residual ethanol. (B) Enriched carboxylate resonance is split in the presence of NAD^+ into two peaks labeled Ox and R (see text for discussion).

FIGURE 5: (Top) Expanded carbonyl carbon region of the 25.15-MHz ^{13}C NMR spectrum of enriched CmLADH in the presence of 3.1 mM NAD^+ at pH 7.4 (taken from Figure 4B). (Bottom) Carbonyl region spectrum of CmLADH (0.74 mM) complexed with 5 mM NADH in pyrophosphate buffer of pH 8.7, the peak labeled R being due to the enriched carboxylate.

the resonance remains unsplit (an exception is noted later) under conditions where it undergoes substantial shift changes, we tentatively conclude that we are observing the resonance due to a *single* enriched carboxylate in each subunit of the enzyme. Further support for this comes from comparing the area (peak integral) of the resonance to the area of the natural abundance envelope (165–185 ppm) due to the unenriched carbonyl, carboxyl, and carboxamido carbons. This ratio was found to be 0.20 (standard deviation of 0.05, average of six samples). The theoretically "expected" ratio is 0.21 based on 90% enrichment and the presence of 418 carbonyl carbons in each subunit (Glu_{21}, Gln_8, Asp_{17}, Asn_8, 373 peptide bonds, one terminal carboxyl; based on data of Jörnvall (1970)). Although this agreement may be fortuitously close and depends on the assumption of similar spin–lattice relaxation times for all these carbons, it is relevant to point out that close agreement also results from a similar analysis in the case of human carbonic anhydrase B. This enzyme has been recently modified by the same enriched $BrAc^-$ reagent at a single active-site histidine (Khalifah et al., 1977; Jeffers et al., 1978). The observed ratio for the enriched carboxylate of that protein was found to be 0.28 (SD of 0.08) by analysis of 20 independent spectra, whereas the theoretically expected ratio is 0.286. Final confirmation is provided by our amino acid analysis of the same CmLADH sample used for NMR, where 0.95 (SD 0.07) and 1.11 (SD 0.06) residues of CmCys were found in two independent hydrolyses.[2] Since IAc^- uniquely alkylates Cys-46 under similar conditions (Li & Vallee, 1964; Zeppezauer et al., 1975), we conclude that the resonance we observe comes from *S*-[^{13}C]$CmCys_{46}$ LADH. Figure 4A demonstrates also the absence of resonances that could be attributed to carboxymethylated histidines, methionines, or other cysteines. ^{13}C NMR has been previously shown to be a sensitive monitor of nonspecific alkylations (Nigen et al., 1973; Eakin et al., 1975).

^{13}C NMR Spectra of Complexes with Coenzymes. The addition of a small excess (three–fivefold) of NAD^+ to enriched CmLADH leads to rather complex alterations in the ^{13}C NMR spectrum (Figures 4B and 5). The enriched carboxylate resonance apparently splits into two peaks of comparable intensity. One peak occurs upfield of the uncomplexed enzyme at 179.6 ppm and is labeled R. The other peak occurs downfield close to 182.2–182.6 ppm and is labeled Ox. The latter peak is markedly broader than the first (roughly 22 vs. 7 Hz in intrinsic line width) and sometimes appears as an incompletely resolved doublet. In contrast to this situation, the addition of a similar excess of NADH gives rise to a single resonance (Figure 5) whose chemical shift and line width are comparable to the peak labeled R in the spectrum of the CmLADH–NAD^+ complex. These observations suggest that in the NAD^+ experiment some of the coenzyme was reduced to NADH, leading to the presence of both complexes,[3] the Ox peak(s) being due to the true NAD^+ complex. In support of this explanation we have searched for and found in the ^{13}C NMR spectrum peaks that are due to the C-1 and C-2 carbons of ethanol (labeled a-1 and a-2, respectively, in Figure 4) at 58.6 and 17.7 ppm, these peaks being of equal integrated intensity. The residual ethanol most likely persisted through the purification and modification of the enzyme which comes as a suspension in 10% ethanol. Note that the workup included two 500-volume excess dialyses and passage on a Sephadex G-25 column. Our experience with residual ethanol is not unique, as the elimination of traces of ethanol from LADH is a recognized and difficult problem (cf. Taniguchi, 1967). Examination of the UV spectrum of a CmLADH–NAD^+ sample to which trifluoroethanol inhibitor was subsequently added also supported the presence of NADH, as judged by the characteristic absorbance near 325–330 nm (Sund & Theorell, 1963). We estimated that about 0.6 equiv

[2] Based on the ratio of the CmCys peak to each of 13 (reliable) reference amino acids of LADH. Standard deviations refer to this averaging over the values from the different amino acid references in each hydrolysate.

[3] Very similar complications have been recently reported from the catalyzed reaction equilibrium during crystallographic work on productive substrate complexes of the holoenzyme (Plapp et al., 1978).

Table III: Chemical Shift of Enriched Carboxylate of Carboxymethylated LADH[a]

enzyme and complexes	pH	chemical shift[b]
CmLADH	7.4–8.7	180.6
denatured CmLADH	9.8	178.5
CmLADH + NAD^+ (1.4–3.1 mM)	7.4	181.2–181.8[c]
CmLADH + NADH (5 mM)	7.4–8.7	179.6
CmLADH + NAD^+ + 4-iodopyrazole (0.8 mM)	6.8–8.4	177.8
CmLADH + NAD^+ + trifluoroethanol (4 mM)	7.4	180.2
CmLADH + NADH + imidazole (5 mM)	8.7	179.6
CmLADH + AMP (2 mM)	7.4	180.5
CmLADH + ADP-ribose (2 mM)	7.4	179.4

[a] Enzyme carboxymethylated at Cys-46 (see text). NMR samples were 0.6–0.9 mM in subunits. [b] In parts per million downfield from Me_4Si assuming internal dioxane is at 67.40. [c] Broad resonance labeled Ox in Figure 5.

of NADH occurred,[4] although this type of measurement does not easily distinguish between bound and free NADH.

^{13}C NMR Shifts in Inhibitor Complexes. Table III lists the chemical shifts of the enriched carboxylate of CmLADH in various binary and ternary complexes of the enzyme. The coenzyme "fragments" AMP and ADP-ribose produce different shifts. That due to AMP is an almost negligible upfield shift of about 0.1 ppm. ADP-ribose, on the other hand, shifts the CmLADH resonance by about 1 ppm upfield to a position very close to that in the NADH complex. The addition of decanoate to the ADP-ribose complex produces no further alterations. The ternary complex with NADH and imidazole was indistinguishable from that of the binary complex with NADH alone. Trifluoroethanol, a substrate competitive inhibitor (Shore et al., 1974), produces an upfield shift of about 1.5 ppm when added to the NAD^+ complex, the comparison being made with respect to the Ox peak. The R peak in this ternary complex was considerably reduced, appearing as a possible shoulder to the observed resonance. A similar but full elimination of the R peak of the NAD^+ sample occurred upon the addition of the powerful inhibitor 4-iodopyrazole[5] which is known to produce an extremely tight ternary complex with NAD^+ (Theorell & Yonetani, 1963). However, the most remarkable aspect of this complex is the dramatic upfield shift, relative to the Ox peak, of approximately 4 ppm. It is noteworthy that this ternary complex proved impossible to dissociate by repeated dialysis in our hands.

pH Effects on the ^{13}C NMR Shifts. Exploratory experiments were carried out to find any pH dependence of the chemical shift of the carboxylate in CmLADH and some of its complexes. Uncomplexed CmLADH showed no pH dependence in the pH range of 7.3 to 8.7. Attempts to record the spectrum at pH 9.8 in glycine buffer resulted in copious precipitation of the enzyme during the spectral acquisition (13 h at room temperature). The accumulated spectrum showed a resonance at 178.5 ppm. Since this shift coincides with the chemical shift of *S*-carboxymethyl model compounds (Table II), we interpret the result as being due to the resonance of denatured CmLADH. No pH dependence was observed in the NADH complex between pH 7.4 and 8.7, nor in the ternary complex with NAD^+ and 4-iodopyrazole between pH 6.8 and 8.4.

[4] NADH is well known to have a stronger affinity for the enzyme than NAD^+ at this pH (Sund & Theorell, 1963).

[5] The disappearance of the R peak in these complexes provides good indication that the peak came from an equilibrium between different species.

Discussion

Carboxymethylation Characteristics. Horse liver alcohol dehydrogenase contains 14 cysteine residues in each subunit (Jörnvall, 1970). Two of these (Cys-46 and Cys-174) occur in the active site and donate sulfur ligands to the catalytic zinc atom (Bränden et al., 1975). Homologous cysteines to these two apparently occur also in the yeast enzyme (Jörnvall, 1978). It has been found that the same two cysteines can be preferentially reacted with a variety of haloacetyl reagents in both enzymes (Bränden et al., 1975), but the relative reactivity of the two is quite dependent on the reagent and the pH of the reaction (Harris, 1964; Belke et al., 1974). Cys-46 of LADH reacts at pH 7.5 exclusively with IAc^- (Li & Vallee, 1964), with possible minor reaction at Met-40 occurring in the crystalline state (Zeppezauer et al., 1975). Substitution of $IAcNH_2$ for IAc^- leads to a loss of the specificity, and many other cysteines become labeled (Reynolds & McKinley-McKee, 1974).

With the above concerns in mind, we have studied the reaction of LADH with $BrAc^-$, a reagent more readily and economically available in ^{13}C-enriched form (Khalifah et al., 1977), and have attempted to compare it with the previously used IAc^- and $IAcNH_2$. Not surprisingly, the characteristics of modification with $BrAc^-$ and IAc^- are virtually identical and show distinct differences when compared to $IAcNH_2$. Particularly relevant here is the rate of reaction, as the loss of specificity with $IAcNH_2$ is due to its much slower rate of reaction with the active-site residue (Reynolds & McKinley-McKee, 1974). Minor differences have been noted also in the comparison of $BrAc^-$ and IAc^-. The AMP protection seen here agrees with Reynolds & McKinley-McKee (1969), although not with the studies on the crystalline enzyme modification (Zeppezauer et al., 1975). The stimulation of the alkylation with imidazole (Figure 3) has been previously noted with IAc^- alkylation (Evans & Rabin, 1968; Reynolds et al., 1970), but the protection at higher concentrations is much more marked than suggested by the data of Reynolds et al. (1970). This protection is unlikely to be due to complications arising from reaction of $BrAc^-$ with imidazole itself, as this is too slow (Heinrikson et al., 1965). Apparently a second imidazole molecule can bind at the active site other than at the known zinc site (Boiwe & Bränden, 1975).

^{13}C NMR Spectrum of CmLADH. In view of the fact that IAc^- alkylates Cys-46 and that $BrAc^-$ shows similar characteristics, one can reasonably expect Cys-46 to be the site of reaction with 90% bromo[1-^{13}C]acetate. A single resonance is seen, in agreement with this expectation, in the CmLADH enzyme (Figure 4A) whose chemical shift is assignable to an *S*-carboxymethyl carboxylate (cf. Table II), even though its position is clearly perturbed by its protein surroundings. Denaturation of the enzyme normalizes the chemical shift to the value precisely expected from the model compound data. In addition, the integrated intensity of the resonance and the amino acid analysis are both consistent with the labeling of a single cysteine in each subunit.

The covalently introduced carboxylate provides a potential probe of the active site of the (modified) enzyme. It is noteworthy that CmLADH is catalytically active particularly at high pH and towards higher aldehyde substrates (Reynolds & McKinley-McKee, 1970, 1975; Björkhem et al., 1973; Hardman, 1976). Our results demonstrate that the resonance is very sensitive to the binding of coenzymes and inhibitors (Table III), although it appears insensitive so far to active-site ionizations. The understanding of the chemical shift changes induced by ligands is predicated to a significant extent on the

understanding of the chemical shift of the uninhibited CmLADH, where it occurs about 2 ppm *downfield* from its expected (unperturbed) position.

One obvious possibility for this abnormal shift is raised by the crystallographic studies (Zeppezauer et al., 1975) which have been interpreted to indicate continued ligation of the sulfur of Cys-46 to the catalytic zinc, despite its conversion from a thiolate to a thioether. The effects of such an S-coordination on the nearby carboxylate carbon resonance have been recently examined through model compound studies (Khalifah, 1978). These studies suggest that an *upfield* shift of about 1 ppm is to be expected from such S-coordination.[6] Although the ^{13}C NMR results appear to exclude such a structure in uninhibited CmLADH, it should be kept in mind that compensating interactions with protein side chains may occur to mask a present upfield shift. The presence of a *direct* zinc–carboxylate coordination, a possibility raised earlier (Reynolds & McKinley-McKee, 1975), cannot be invoked here. Such an interaction has recently been deduced in carboxymethylated human carbonic anhydrase B where it was shown to lead to an *upfield* shift of about 1 ppm (Jeffers et al., 1978).

Speculation on the observed 2-ppm downfield shift of CmLADH must thus center on interactions of the introduced carboxylate with protein side chains in its immediate vicinity. Carboxylate resonances are known to be particularly sensitive to electric field effects that are both distance and orientation dependent (Batchelor, 1975; Batchelor et al., 1975). It is thus interesting that two potentially charged side chains have been identified near the introduced carboxylate in CmLADH, these being Arg-369 and Glu-68 (Zeppezauer et al., 1975). The proximity of Glu-68 to the carboxylate may account for the downfield shift. However, no protonation of the enriched carboxylate results from this potential interaction, since such protonation would have led to an upfield shift of about 3–4 ppm (Table II), which is not observed. Some caution should be exercised in interpreting the NMR results in terms of the reported crystal structure of CmLADH, as the latter was carried out on enzyme apparently inhibited at the zinc by a halide anion.[7]

Binding of Coenzymes and Analogues. The enriched carboxylate has proved to be especially sensitive to the binding of coenzymes in binary and ternary complexes. The simplest tight-binding coenzyme analogue is AMP which produces a negligible change in the carboxylate chemical shift.[8] Apparently it is much too distant from the zinc coordination sphere (Zeppezauer et al., 1975). This is not the case with ADP-ribose which produces an upfield shift of about 1 ppm. The binding of this coenzyme "fragment" apparently brings either the second phosphate of the pyrophosphate group or, more likely, the nicotinamide ribose into close proximity with the carboxylate. These possibilities may be resolvable by a study of complexes with ADP. However, regardless of the origin of the perturbation, it is significant that similar changes are produced by both NADH and ADP-ribose. This suggests that in the NADH complex, the enriched carboxylate does not necessarily interact with the nicotinamide ring portion and that both molecules are similarly oriented with respect to their common atoms, i.e., the ADP-ribose moeity. This is in excellent agreement with crystallographic findings on LADH (Samama et al., 1977) and CmLADH (Bränden, 1977). This is *not* the case with all NADH analogues. For example, pyridine adenine dinucleotide has been recently found to be oriented in a unique way, with rotation around the pyrophosphate group leading to a different placement of the nicotinamide ribose and the pyridine ring (Samama et al., 1977). The ^{13}C NMR study of the enriched carboxlate thus offers promise of distinguishing between these two alternative modes of binding independently of crystallographic methods.

In view of the above, it seems that the NADH-induced conformational change in the enzyme (Bränden, 1965; Bränden et al., 1975) is unlikely to be associated with the two modes of coenzyme binding originating in rotation around the pyrophosphate group. Rather, they must be triggered at the nicotinamide ring of NADH *without* alteration in the rest of the coenzyme binding. The binding of the oxidized coenzyme NAD^+ remains much more obscure and has not been studied crystallographically in simple binary complexes with LADH or CmLADH. Our ^{13}C NMR results show that NAD^+ produces an entirely different pattern of shifts in binary and ternary complexes. NAD^+ alone produces a *downfield* shift of about 1 ppm, perhaps reflecting a different orientation for NAD^+ as compared to either ADP-ribose or NADH. In addition, it is quite possible that NAD^+ may bind in more than one orientation in view of the significant broadening and/or splitting of the carboxylate resonance (Ox peak of Figure 5). These could be related to the pyridine adenine dinucleotide binding mode. The unusual broadening and/or splitting seen in the NAD^+ case is unlikely to be due to either slow interconversion of ternary substrate complexes ($E–NAD^+–Alc \rightleftarrows E–NADH–Ald$) or to processes involving abortive E–NADH–Alc complexes, both of which could arise from the residual ethanol in the NMR samples. Neither the residual alcohol signals nor the enriched carboxylate peak in the NADH complex appears unusually broadened in a manner suggesting the occurrence of such complications.

The addition of 4-iodopyrazole apparently results in the formation of an extremely tight complex with NAD^+ and CmLADH. This is consistent with previous observations on complexes of pyrazoles with LADH (Theorell & Yonetani, 1963; Theorell et al., 1969) and CmLADH (Reynolds & McKinely-McKee, 1975). The precise mode of binding of pyrazoles has not been elucidated, however. The effect of 4-iodopyrazole on the chemical shift of the carboxylate in the NAD^+–CmLADH complex is very dramatic, the 4-ppm shift being reminiscent of protonation effects on the carboxylate (Table II). The actual chemical shift in this ternary complex is only a little upfield of the shift of the carboxylate resonance in either denatured CmLADH (Table III) or in *S*-carboxymethyl model compounds (Table II). Since metal coordination at either the carboxylate (Jeffers et al., 1978) or the thioether sulfur[6] (Khalifah, 1978) produces upfield shifts in model compounds, it is possible that the carboxylate has become oriented toward the metal to permit either type of interaction. Another possibility is for the carboxylate to interact with the positively charged pyridinium nitrogen of the nicotinamide ring

[6] The upfield shift in the carboxylate resonance seen upon metal coordination at the nearby thioether sulfur probably arises from orientation- and distance-dependent electric field effects (Khalifah, 1978). It is quite possible that if active-site interactions constrain the rotation of the carboxymethyl group so that the carboxylate is oriented away from the metal, then no upfield perturbation will result from metal coordination.

[7] A spherical electron density in CmLADH was observed at the zinc open ligand position and was assigned to the iodide released by the reaction with IAc^- (Zeppezauer et al., 1975). Since the enzyme crystals were apparently dialyzed to remove excess reagent, it is also possible that the density was due to the chloride in the soaking buffer (0.05 M Tris-HCl).

[8] This is not inconsistent with the protective effect of AMP in the carboxymethylation reaction (cf. Figure 2), as protection probably occurs by inhibiting *reversible* binding of haloacetates and the carboxymethyl group assumes a different position after the *covalent* reaction (cf. Figure 16 of Bränden et al., 1975, and discussion therein).

with possibilities of ring-current shifts. In all cases, the results suggest a different orientation of the coenzyme with respect to the carboxylate marker.

In view of the above observations and considerations, it may be significant that the addition of trifluoroethanol, a substrate analogue (Shore et al., 1974), to the CmLADH–NAD^+ complex produces a carboxylate chemical shift close to that of the NADH binary complex (Table III). As suggested by Parker et al. (1978), the alcohol substrate is important for orienting the NAD^+ coenzyme in the proper conformation necessary for catalysis in CmLADH, presumably one similar to that of NADH. Further insights into the mechanistic details of the enzyme could not, unfortunately, be obtained by studies of active site ionizations using this probe. The carboxylate chemical shift has proven so far to be insensitive to pH changes in the active site in the presence and absence of some of the ligands. The nature of the proton-linked group(s) in the mechanism continues to elude identification, despite much intensive efforts (DeTraglia et al., 1977; Wolfe et al., 1977; Coates et al., 1977; Parker et al., 1978; Kvassman & Pettersson, 1978). Further structural studies of the active site are clearly needed, especially in view of the inherent ambiguities in the recent determination of the crystal structure of the holoenzyme in the presence of competent substrates (Plapp et al., 1978).

Acknowledgments

We are particularly indebted to Professor Warren C. Jones for the amino acid analyses, to Pamela Jeffers Morley for invaluable assistance during this project, and to Professor R. Bruce Martin for helpful discussions.

References

Baici, A., & Luisi, P. L. (1977) *J. Mol. Biol. 114*, 267–279.
Batchelor, J. G. (1975) *J. Am. Chem. Soc. 97*, 3410–3415.
Batchelor, J. G., Feeny, J., & Roberts, G. C. K. (1975) *J. Magn. Reson. 20*, 19–38.
Belke, C. J., Chin, C. Q. C., & Wold, F. (1974) *Biochemistry 13*, 3418–3420.
Bernhard, S. A., Dunn, M. F. Luisi, P. L., & Schack, P. (1970) *Biochemistry 9*, 185–192.
Björkhem, I., Jörnvall, H., & Zeppezauer, E. (1973) *Biochem. Biophys. Res. Commun. 52*, 413–420.
Boiwe, T., & Bränden, C.-I. (1977) *Eur. J. Biochem. 77*, 173–179.
Bonnichsen, R. K. (1950) *Acta Chem. Scand. 4*, 715.
Bränden, C.-I. (1965) *Arch. Biochem. Biophys. 112*, 215–217.
Bränden, C.-I. (1977) *Biochem. Soc. Trans. 5*, 612–615.
Bränden, C.-I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 11*, 103–190.
Coates, J. H., Hardman, M. J., Shore, J. D., & Gutfreund, H. (1977) *FEBS Lett. 84*, 25–28.
Dalziel, K. (1975) *Enzymes, 3rd Ed. 11*, 1–60.
DeTraglia, M. C., Schmidt, J., Dunn, M. F., & McFarland, J. T. (1977) *J. Biol. Chem. 252*, 3493–3500.
Drott, H. R. (1978) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Williamson, J. R., Drott, H., & Chance, B., Eds.) Vol. 2, pp 87–101, Academic Press, New York, NY.
Drott, H. R., Harvey, R. A., Mildvan, A. S., & Young, J. M. (1975) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Williamson, J. R., Drott, H., & Chance, B., Eds.) Vol. 2, pp 119–122, Academic Press, New York, NY.
Drum, D. E., & Vallee, B. L. (1970) *Biochem. Biophys. Res. Commun. 41*, 33–39.
Drum, D. E., Harrison, J. H., Li, T.-K., Bethune, J. L., & Vallee, B. L. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 1434–1440.
Drum, D. E., Li, T.-K., & Vallee, B. L. (1969) *Biochemistry 8*, 3783–3791.
Dunn, M. F. (1975) *Struct. Bonding (Berlin) 23*, 61–122.
Eakin, R. T., Morgan, L. O., & Matwiyoff, N. A. (1975) *Biochemistry 14*, 4538–4543.
Ernst, R. R., & Anderson, W. A. (1966) *Rev. Sci. Instrum. 37*, 93.
Evans, N., & Rabin, B. R. (1968) *Eur. J. Biochem. 4*, 548–554.
Farrar, T. C., & Becker, E. D. (1971) *Pulse and Fourier Transform NMR*, Chapter 4, Academic Press, New York, NY.
Gurd, F. R. N. (1972) *Methods Enzymol. 25*, 424–438.
Hadron, M., John, V. A., Meir, F. F., & Dutler, H. (1975) *Eur. J. Biochem. 54*, 65–73.
Hardman, M. J. (1976) *Eur. J. Biochem. 66*, 401–404.
Harris, I. (1964) *Nature (London) 203*, 30.
Heinrikson, R. L., Stein, W. H., Crestfield, A. M., & Moore, S. (1965) *J. Biol. Chem. 240*, 2921–2934.
Jeffers, P. K., Sutherland, W. M., & Khalifah, R. G. (1978) *Biochemistry 17*, 1305–1312.
Jörnvall, H. (1970) *Eur. J. Biochem. 16*, 25–40.
Jörnvall, H. (1978) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Williamson, J. R., Drott, H. R., & Chance, B., Eds.) Vol. 2, pp 145–155, Academic Press, New York, NY.
Khalifah, R. G. (1978), *Inorg. Chim. Acta*, in press.
Khalifah, R. G., Strader, D. J., Bryant, S. H., & Gibson, S. M. (1977) *Biochemistry 16*, 2241–2247.
Kvassman, J., & Pettersson, G. (1978) *Eur. J. Biochem. 87*, 417–427.
Li, T.-K., & Vallee, B. L. (1963) *Biochem. Biophys. Res. Commun. 12*, 44–49.
Li, T.-K., & Vallee, B. L. (1964) *Biochemistry 3*, 869–873.
Mildvan, A. S., & Weiner, H. (1969) *J. Biol. Chem. 244*, 2465–2475.
Nigen, A. M., Keim, P., Marshall, R. C., Morrow, J. S., Vigna, R., & Gurd, F. R. N. (1973) *J. Biol. Chem. 248*, 3724–3732.
Parker, D. M., Hardman, M. J., Plapp, B. V., Holbrook, J. J., & Shore, J. D. (1978) *Biochem. J. 173*, 269–275.
Pietruszko, R., Clark, A., Graves, J., & Ringold, H. J. (1966) *Biochem. Biophys. Res. Commun. 23*, 526.
Plapp, B. V., Eklund, H., & Bränden, C.-I. (1978) *J. Mol. Biol. 122*, 23–32.
Reynolds, C. H., & McKinley-McKee, J. S. (1969) *Eur. J. Biochem. 10*, 474–478.
Reynolds, C. H., & McKinley-McKee, J. S. (1970) *Biochem. J. 119*, 801–802.
Reynolds, C. H., & McKinley-McKee, J. S. (1974) *FEBS Lett. 46*, 83–86.
Reynolds, C. H., & McKinley-McKee, J. S. (1975) *Arch. Biochem. Biophys. 168*, 145–162.
Reynolds, C. H., Morris, D. L., & McKinley-McKee, J. S. (1970) *Eur. J. Biochem. 14*, 14–26.
Samama, J.-P., Zeppezauer, E., Biellmann, J.-F., & Bränden, C.-I. (1977) *Eur. J. Biochem. 81*, 403–409.
Shore, J. D., Gutfreund, H., Brooks, R. L., Santiago, D., & Santiago, P. (1974) *Biochemistry 13*, 4185–4191.
Sloan, D. L., Young, J. M., & Mildvan, A. S. (1975a) in *Structure and Conformation of Nucleic Acids and Protein–Nucleic Acid Interactions* (Sundaralingam, M., & Rao,

S. T., Eds.) pp 397–406, University Park Press, Baltimore, Md.
Sloan, D. L., Young, J. M., & Mildvan, A. S. (1975b) *Biochemistry 14*, 1998–2008.
Sund, H., & Theorell, H. (1963) *Enzymes, 3rd Ed. 7*, 25.
Sytkowski, A. J., & Vallee, B. L. (1978) *Biochemistry 17*, 2850–2857.
Taniguchi, S. (1967) *Acta Chem. Scand. 21*, 1511–1518.
Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553.
Theorell, H., Yonetani, T., & Söjberg, B. (1969) *Acta Chem. Scand. 23*, 255–260.
Thurman, R. G., Yonetani, T., Williamson, J. R., & Chance, C., Eds. (1973) *Alcohol and Aldehyde Metabolizing Systems*, Vol. 1, Academic Press, New York, NY.
Thurman, R. G., Williamson, J. R., Drott, H. R., & Chance, B., Eds. (1977) *Alcohol and Aldehyde Metabolizing Systems*, Vol. 3, Academic Press, New York, NY.
Thurman, R. G., Williamson, J. R., Drott, H. R., & Chance, B., Eds. (1978) *Alcohol and Aldehyde Metabolizing Systems*, Vol. 2, Academic Press, New York, NY.
Ulmer, D. D., & Vallee, B. L. (1971) *Adv. Chem. Ser. No. 100*, 187–218.
Weidig, C. F., Halvorson, H. R., & Shore, J. D. (1977) *Biochemistry, 16*, 2916–2922.
Wolfe, J. K., Weidig, C. F., Halvorson, H. R., Shore, J. D., Parker, D. M., & Holbrook, J. J. (1977) *J. Biol. Chem. 252*, 433–436.
Young, J. M., & Mildvan, A. S. (1978) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Williamson, J. R., Drott, H. R., & Chance, B., Eds.) Vol. 2, pp 109–117, Academic Press, NY.
Zeppezauer, E., Jörnvall, H., & Ohlsson, I. (1975) *Eur. J. Biochem. 58*, 95–104.