Magnetic Resonance Studies of the Geometry of Bound Nicotinamide Adenine Dinucleotide and Isobutyramide on Spin-Labeled Alcohol Dehydrogenase†

Donald L. Sloan* and Albert S. Mildvan

ABSTRACT: Inactivation of yeast alcohol dehydrogenase by stoichiometric concentrations of a paramagnetic iodoacetamide analog (I·) results in a spin-labeled complex (EI·) in which the unpaired electron is highly immobilized. The effect of the bound paramagnetic species on the longitudinal relaxation rate ($1/T_1$) of water protons is enhanced by a factor of 6. Enhancement is decreased 14% by NADH and 12% by NAD^+. It is increased 8% by ethanol, 6% by isobutyramide, and 8% by acetaldehyde indicating that substrates bind in the absence of coenzymes and alter the spin–H_2O interaction. The dissociation constants of substrates and coenzymes agree with their K_m and K_I values. From $1/T_1$ measurements of six protons of NADH and the methyl protons of isobutyramide at 100 and 220 MHz, and of the phosphorus of NADH at 40.5 MHz, the distances between the enzyme-bound spin and these nuclei were determined in the binary EI·NADH and ternary EI·NADH–isobutyramide complexes. The mean distances from the spin to the protons of bound NADH and isobutyramide range from 8 to 14 Å. On forming the ternary complex, the distance from the spin to the dihydropyridine C_2 proton and to the adenine C_2 and C_8 protons decreased by 0.7 Å, and the distance to the C_1 proton of the adenosine ribose increased by 1.3 Å. These changes in distance suggest a conformation change of bound NADH when isobutyramide binds. The conformation of NADH on yeast alcohol dehydrogenase in solution from nuclear magnetic resonance (nmr) studies is open, with adenine-ribose in an anti relationship, and is very similar to that of ADP-ribose on liver alcohol dehydrogenase from the crystallographic studies of Branden *et al.* (1973, *Proc. Nat. Acad. Sci. U. S. 70*, 2439). Comparison of the nmr model of NADH bound to alcohol dehydrogenase with the crystallographic models of NAD^+ bound to lactate and malic dehydrogenases indicates that the pyridine and adenine rings are approximately perpendicular in all cases, but that differences occur in the dihedral angle between the pyridine C_6 and the ribose ring oxygen of the bound coenzyme. A combination of the nmr and crystallographic data on alcohol dehydrogenase defines the orientation of the spin-label on the protein and suggests that the substrate is directly coordinated to the active-site zinc.

The geometric arrangement and conformations of enzyme-bound substrates often provide valuable clues to the mechanism of enzyme action. Extending the early observations of Boyer and Theorell (1956), Velick first established the conformation of NADH on several dehydrogenases to be open, as judged by the absence of fluorescence transfer from adenine to dihydropyridine (Velick, 1958, 1961). Velick's results also implicated a fixed coenzyme conformation for each enzyme complex, and suggested that one conformation might be common to a class of dehydrogenases. These conclusions were confirmed and extended by X-ray diffraction studies of the crystalline NAD^+–lactate dehydrogenase complex (Chandrasekhar *et al.*, 1973) and the NAD^+–malic dehydrogenase complex (Webb *et al.*, 1973). Nuclear magnetic resonance (nmr) studies of NAD^+ and NADH bound to various dehydrogenases in solution (Oppenheimer *et al.*, 1971; Lee *et al.*, 1973) have also yielded results consistent with Velick's original proposal. However, as has often been pointed out (Jardetzky, 1964; Mildvan and Weiner, 1969a), diamagnetic effects of enzymes on the nmr spectra of substrates are usually difficult to interpret in terms of structure. Hence an independent nmr approach was chosen, utilizing a bound paramagnetic probe, to examine the geometry of NADH and substrates on a dehydrogenase. Such studies have been previously made with a paramagnetic analog of NAD^+ to estimate distances between bound NAD^+ and substrates on dehydrogenases (Mildvan and Weiner, 1969a,b; Mildvan *et al.*, 1972) and with spin-labels covalently bound to lysozyme and substrate analogs to determine various enzyme–substrate distances (Wien *et al.*, 1972). Wien's distances agreed with corresponding distances in the crystallographic model of lysozyme (Phillips, 1967). The present studies make use of a spin-label covalently bound to the sulfhydryl group of the cysteine residue 43 (Jornvall, 1973) near the active site of yeast alcohol dehydrogenase (Harris, 1964). The enzyme from yeast was chosen because a one site stoichiometric modification with iodoacetate has been established (Harris, 1964) under specific experimental conditions (see Twu *et al.*, 1973) and because, unlike the liver enzyme from which dissociation of NADH is slow (3 sec^{-1}, Theorell and Chance (1951)), the rate of dissociation of the yeast enzyme–NADH complex ($\geq$491 sec^{-1}, Nygaard and Theorell (1955)) is rapid enough for the nmr experiment which requires fast exchange between free and bound coenzyme. A preliminary report of this work has been published (Sloan and Mildvan, 1973).

Experimental Section

Materials. Yeast alcohol dehydrogenase was obtained in the lyophilized form (lot 15420) from Boehringer Mannheim. Extraneous salt and sucrose were removed from solutions of the protein by Sephadex G-25 column chromatography before

† From the Institute for Cancer Research, Fox Chase Center for Cancer and Medical Sciences, Philadelphia, Pa. 19111. *Received September 24, 1973.* This work was supported by U. S. Public Health Service Grants AM-13351, CA-06927, and RR-00542 (for use of nmr equipment located at the University of Pennsylvania), Grant RR-05539 from the National Institutes of Health, Grant GB-27739X (previously Grant GB-8579) from the National Science Foundation, and by an appropriation from the Commonwealth of Pennsylvania.

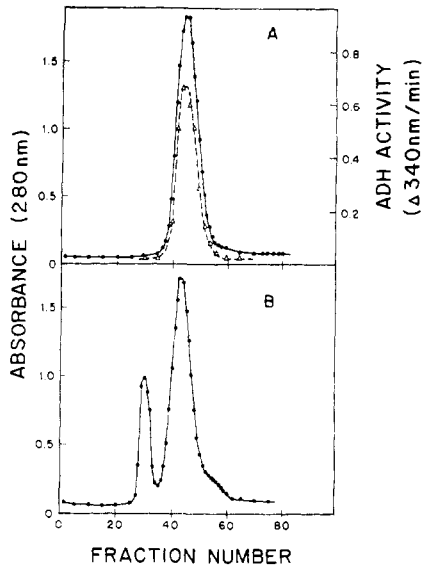

FIGURE 1: Sephadex G-200 elution profiles of alcohol dehydrogenase before (A) and after (B) incubation with iodoacetamide spin-label. The activities of the eluent fractions, determined as described in the text, are denoted as open triangles. Elution conditions: 0.05 M potassium phosphate buffer (pH 7.5), 4°.

each experiment. The enzyme was judged to be pure by its behavior on Sephadex G-200 (Figure 1A) and by its specific activity (250 units/mg) when assayed under the conditions of Vallee and Hoch (1955). Protein concentrations were determined spectrophotometrically using an extinction coefficient at 280 nm of $E_{cm}^{1\%} = 12.6$ and a molecular weight of 150,000 (Hayes and Velick, 1954).

The iodoacetamide spin-label [4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinooxyl] was obtained from Synvar Associates. NADH and NAD^+ were purchased in precalibrated vials from Sigma Chemicals. Isobutyramide was an Eastman Organic reagent. All other chemicals were of the highest purity available, and all solutions of the above were freed of any metal contamination by elution through Chelex-100 (Na^+ form, Bio-Rad).

Kinetic Studies. The K_I of isobutyramide with respect to acetaldehyde was determined by double reciprocal plots of activity at five levels of substrate and four levels of the inhibitor in the presence of 3.3 mM NADH in 0.05 M potassium phosphate at pH 7.5. Activity was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm following the addition of 2 μl of 7.7 mg/ml yeast alcohol dehydrogenase to 3 ml of assay mixture.

Preparation of Spin-Labeled Enzyme. The spin-labeled alcohol dehydrogenase was prepared by reacting 3.75 mg/ml of enzyme (0.1 mM active sites) with a concentration of the iodoacetamide analog equivalent to the concentration of active sites, in potassium phosphate (pH 7.5) at 25°. An analogous solution free of inhibitor and one containing iodoacetamide were run concurrently and used as activity standards. The loss of alcohol dehydrogenase activity in the incubation mixture, which paralleled the appearance of the water proton relaxation enhancement due to the bound spin-label, was complete after 8 hr. The spin-labeled tetramer was then separated from higher molecular weight aggregates by centrifugation and by gel filtration on Sephadex G-200 (Figure 1B). Its uv absorption spectrum was indistinguishable from that of the unmodified enzyme.

For measurements of relaxation rates of substrate and coenzyme protons, spin-labeled enzyme was exchanged into D_2O by first lyophilizing the protein to dryness and then dissolving the protein in D_2O. The loss of soluble protein through denaturation after several exchanges was less than 10%. For measurements of the phosphorus relaxation rate, the system was dissolved in 50% D_2O to permit use of the heteronuclear internal field frequency lock of the XL-100 instrument.

Magnetic Resonance Measurements. Pulse nmr measurements at 24.3 MHz of the longitudinal relaxation rate of water protons at 23° were made as previously described (Mildvan and Weiner, 1969a,b; Mildvan *et al.*, 1972). A period of 4 min elapsed between sample insertion and the null point reading in all cases to allow for complete temperature equilibration. Dilution effects were attenuated by titrating a sample of enzyme with buffer (0.05 M phosphate (pH 7.5)), noting the linear effect on the relaxation rate and subtracting this from enhancement perturbations made by the coenzymes and substrates.

The electron paramagnetic resonance (epr) spectra of the spin-labeled enzyme and its substrate complexes were obtained on a Varian E-4 epr spectrometer equipped with a nitrogen-flow temperature controller at 9.15 GHz and at 23°. The spectra were stored in a Fabritek Model 1074 computer for derivation of difference spectra and for peak integration.

The longitudinal ($1/T_1$) relaxation rates of 9 nuclei of NADH and isobutyramide were measured with an average error of 7% at 23° by pulsed Fourier transform nmr (McDonald and Leigh, 1973) at 40.5 MHz (phosphorus) and at 100 and 220 MHz (protons) using the Varian XL-100 and HR-220 instruments. A complete description of the experimental procedures has been published elsewhere (Nowak *et al.*, 1973; Fung *et al.*, 1973). Typically 8–10 points, reflecting a corresponding number of intervals between the demagnetizing and monitoring pulses, were used for each exponential plot. Transverse ($1/T_2$) relaxation rates were obtained with an average error of ±20% from measurements of the width of the resonance signal at half-height (protons at 220 MHz) and with an error of ±11% by the pulsed Fourier transform method (protons at 100 MHz and phosphorus at 40.5 MHz).

Calculations of Correlation Times and Distances. Paramagnetic contributions to the longitudinal ($1/T_{1p}$) and transverse ($1/T_{2p}$) relaxation rates were calculated by subtracting the diamagnetic contributions as previously described (Mildvan and Engle, 1972). The diamagnetic corrections to the relaxation rates were measured with NADH and isobutyramide bound to two analogous diamagnetic systems with identical results. These diamagnetic systems were the enzyme inactivated with iodoacetamide, and the diamagnetic amine derived by reducing EI·[1] with a tenfold excess of ascorbate. The $1/T_{1p}$ and $1/T_{2p}$ values were normalized by the concentration ratio (f) = (spin-labeled binding site)/(ligand), as described by Luz and Meiboom (1964). The distances (r) from the unpaired electron to the nuclei of NADH and isobutyramide were calculated from the dipolar term of the Solomon–Bloembergen equation as described by Mildvan and Engle (1972). In eq 1 C

$$r(\text{in Å}) = C[T_{1M}f(\tau_c)]^{1/6} \quad (1)$$

is a product of physical constants numerically equal to 539 and 399 for the interaction of nitroxides with protons and phosphorus, respectively, T_{1M} is the relaxation time of a nucleus of the bound coenzyme or substrate and is equal to fT_{1p} in the limit of fast exchange (Fung *et al.*, 1973; Nowak

[1] Abbreviations used are: EI·, spin-labeled yeast alcohol dehydrogenase; ADP-ribose, adenosine diphosphate ribose.

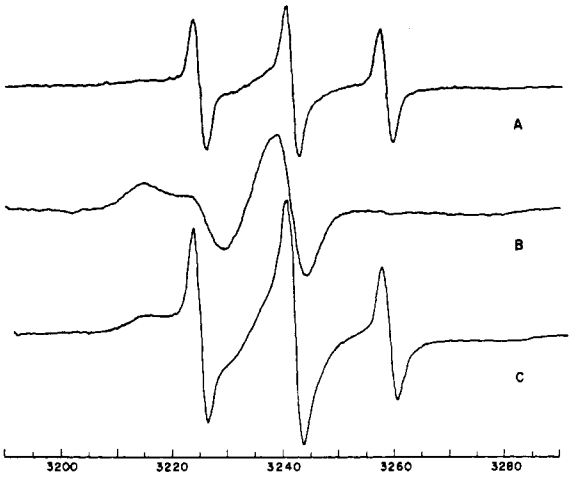

FIGURE 2: Epr spectra of the spin-labeled alcohol dehydrogenase before (C) and after (A and B) Sephadex G-200 chromatography. The spin-labeled tetrameric enzyme (0.5 mM, spectrum B) was five times as concentrated as the labeled aggregate (0.1 mM, spectrum A) in order to simulate their relative concentrations in the incubation mixture (spectrum C). Conditions: 0.05 M potassium phosphate (pretreated with Chelex-100), pH 7.5, 23°.

et al., 1973) as will be shown to be true in the present case, and $f(\tau_c)$, the correlation function, is given by

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_S^2\tau_c^2} \quad (2)$$

In eq 2, τ_c is the correlation time for dipolar interaction, ω_I is the nuclear precession frequency, and ω_S is the electron precession frequency. The correlation times (τ_c) and correlation functions ($f(\tau_c)$) were evaluated by three independent techniques, the frequency dependence of $1/fT_{1p}$, the T_{1p}/T_{2p} ratio, and the line width of the epr spectrum of the bound nitroxide. Because of the greater error levels in $1/T_2$ at 220 MHz and in the epr line widths, the $f(\tau_c)$ values calculated from these data were not used in the final distance calculations.

Results and Discussion

Epr Studies. The epr spectrum of yeast alcohol dehydrogenase, inactivated by the iodoacetamide spin-label, consists of two overlapping resonances (Figure 2C): a broad signal due to a highly immobilized spin, and a narrower signal due to a partially immobilized spin as was observed for the enzyme from liver (Spallholz and Piette, 1972). Upon elution through a calibrated Sephadex G-200 column two protein fractions were resolved (Figure 1B). The epr spectrum of the highly immobilized spin (Figure 2B) was associated with the protein which migrated with the native tetrameric molecular weight (150,000) while the partially immobilized spin (Figure 2A) was associated with an aggregate of higher molecular weight (~310,000). All subsequent studies were carried out with the inactivated tetramer, hereafter referred to as EI·.

Double integration of the epr spectrum of EI·, using the iodoacetamide spin-label reagent as a standard, indicated that complete inactivation of the enzyme occurred at a stoichiometry of 2.8 ± 0.3 spins per enzyme tetramer at pH 7.5. This result is in agreement with the number of binding sites for NADH (2.7 ± 0.3), found with the native enzyme (Dickinson, 1970), and for another active-site specific reagent, butyl isocyanate (Twu and Wold, 1973).

NADH and, to a lesser extent, NAD^+ caused a narrowing of the epr spectrum of EI· (Figure 3) indicating that the co-

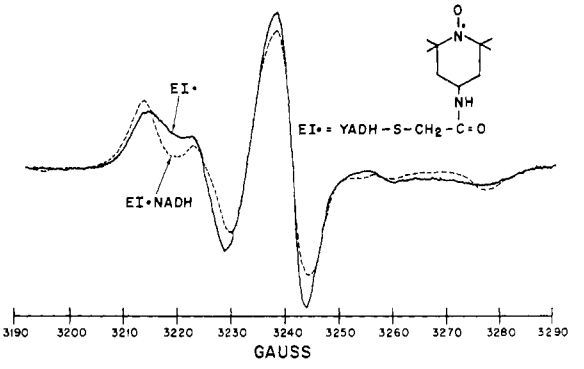

FIGURE 3: Epr spectra of EI· with (- - - - -) and without (—) saturating amounts of NADH (5 mM) at 23°. Conditions were otherwise as described in Figure 2.

enzymes could still bind to the inactive enzyme and bring about an increase in the mobility of the spin-label. The substrate acetaldehyde and its analog isobutyramide produced small changes in the epr spectrum consistent with binding to EI·, while ethanol produced no detectable change. However, the binding of ethanol, acetaldehyde, and isobutyramide to EI· was clearly detected by changes in $1/T_1$ of water protons (*vide infra*). Within experimental error the effects of substrates and coenzymes on the epr spectrum of EI· were additive.

Water Proton Relaxation Studies. When covalently bound to alcohol dehydrogenase, the spin-label was six times as effective as the unbound nitroxide radical in increasing the longitudinal relaxation rate ($1/T_1$) of water protons, *i.e.*, the enhancement factor (ϵ, as defined in Table I) was 6.0. This value of ϵ is of the same order as that found for a paramagnetic analog of NAD^+ bound to liver alcohol dehydrogenase (ϵ = 12, Mildvan and Weiner, (1969a,b)) or to malic dehydrogenase (ϵ = 5, Mildvan *et al.* (1972)) and is ascribed to hindered rotation of water molecules near the unpaired electron.

The addition of substrates or substrate analogs to EI· (Figure 4) caused a 6–12% increase in the enhancement factor. These increases in ϵ, together with the very small changes in the epr spectrum, indicate that further immobilization of water protons near the unpaired electron has resulted from substrate binding.

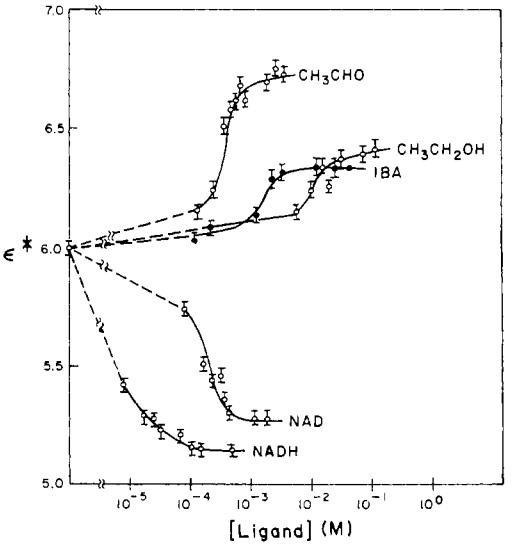

FIGURE 4: Titration of EI· with substrates and coenzymes measuring the enhancement (ϵ^*) of $1/T_1$ of water by the pulse-sequence method of Carr and Purcell (1954). Conditions: 0.05 M potassium phosphate (pretreated with Chelex-100), pH 7.5, 23°.

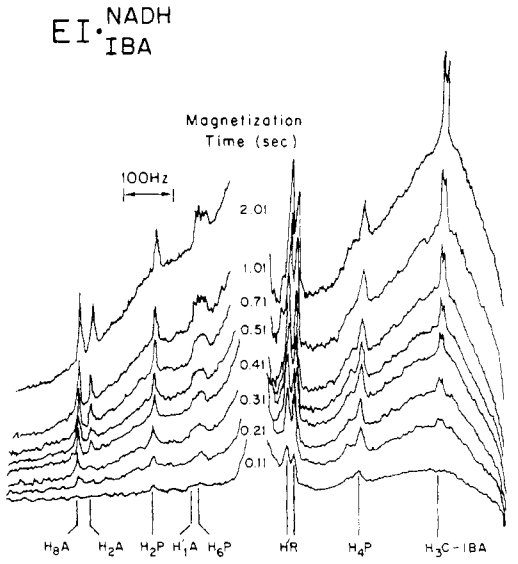

FIGURE 5: T_1 measurement of the protons of NADH (15 mM) and isobutyramide (10 mM) in the presence of EI· (1200 μM in enzyme-bound spins) by the partially relaxed Fourier transform method of McDonald and Leigh (1973). The assignments are from Jardetzky and Wade-Jardetzky (1966), Oppenheimer *et al.* (1971), and Mildvan and Weiner (1969a). Conditions: 0.05 M phosphate in D_2O (pD 7.5), 23 ± 1°. Initial sample volume was 0.40 ml.

Unlike the substrates, the coenzymes caused a 12–14% decrease in enhancement (Figure 4). As pointed out elsewhere (Mildvan and Engle, 1972) such decreases in ϵ result from a decrease in the access of water protons to the bound spin or from a decrease in the correlation time τ_c for spin–proton dipolar interaction. A comparison of the epr spectra and the $1/T_1$ data suggests that both of these effects are operative.

Titrations of the effects of substrates, inhibitors, and coenzymes on $1/T_1$ of water in the presence of EI· (Figure 4) yield dissociation constants (Table I) in reasonable agreement with K_M and K_I values obtained by kinetic studies on the active enzyme and with reported K_D values (Sund and Theorell, 1963). This agreement indicates that inactivation of yeast alcohol dehydrogenase by chemical modification of a thiol group has little or no effect on the affinity of the enzyme for its substrates.

TABLE I: Enhancements of Water Proton Relaxation Rate and Dissociation Constants of EI· Ligand Complexes.

Ligand	ϵ_T/ϵ_b[a,b]	Spin-Labeled E K_D (μM)	Unmodified E[c] K_D (μM)	Unmodified E[c] K_M or K_I (μM)
NADH	0.86	6	20	14
NAD^+	0.88	100	195	200
CH_3CHO	1.12	290	370	200
CH_3CH_2OH	1.08	7500	19,000	16,000
IBA[d]	1.06	1500		3,300–11,000[e]

[a] ϵ_T/ϵ_b = ratio of the enhancement of the water proton relaxation rate in solutions of the ternary ER· ligand complex (ϵ_T) to that of the binary ER· complex (ϵ_b). As discussed in the text, ϵ_b = 6.0. [b] ϵ_b is defined as the ratio of the paramagnetic contribution to the relaxation rate of water in the presence of EI· to that in the presence of the iodoacetamide analog (I·) alone. [c] From data presented in Sund and Theorell (1963). [d] A K_D value of 5000 μM has been determined for isobutyramide (IBA) and alcohol dehydrogenase from horse liver at pH 7 (Winer and Theorell, 1960). [e] Positive curvature in double reciprocal plots.

TABLE II: Effect of EI· on the Relaxation Rates of the Protons and Pyrophosphate Phosphorus of NADH and the Methylene Protons of Isobutyramide in Binary (EI·NADH) and Ternary (EI·NADH·IBA) Complexes.[a]

Nucleus	ν_I (MHz)	Binary $1/fT_{1p}$[b]	Binary $1/fT_{2p}$	Ternary $1/fT_{1p}$	Ternary $1/fT_{2p}$
H_8Ad	100	6.7	78.2	9.9	72.1
	220			<1.0	65.6[c]
H_2Ad	100	2.1	101.9	2.5	145.7
	220			<1.0	75.5
H'Ad	100	6.0	178.2	1.9	186.1
	220			<1.0	66.0
P-P	40.5	3.9	37.5	2.3	108.7
H_2Py	100	11.2	126.0	17.0	152.6
	220			5.0	59.5
H_4Py	100	46.6	562.0	35.1	524.8
	220			12.3	107.0
H_6Py	100	23.4	363.0	17.5	1204.0
	220			7.8	72.0[c,d]
H_3C–IBA	100			3.6	236.5
	220			1.2	62.5

[a] Uncertainty in the T_1 measurements is ±7%. The average error in $1/fT_{1p}$ values is ±20%. Errors in $1/fT_{2p}$ are ±15% at 100 MHz and ±28% at 220 MHz. [b] Concentration factor for protons of NADH (f) = 0.0865, for methylene protons of IBA (f) = 0.1294, for phosphorus of NADH (f) = 0.0433. [c] T_2 values at 220 MHz were obtained by measuring the width of the resonance signal at half-height in a sample containing 13% of the labeled aggregate described in the text. [d] The unusually high ratio of $(1/fT_{2p}$ (100 MHz))/$(1/fT_{2p}$ (220 MHz)) is due in part to the greater errors in $1/fT_{2p}$(220 MHz).

NADH and Isobutyramide Relaxation Studies. The geometry of the NADH bound to EI· was studied by measuring the effects of the unpaired electron of the nitroxide radical on the relaxation rates of six carbon-bound protons (Figure 5) and the unresolved pyrophosphate phosphorus atoms of the coenzyme (Figure 6), and the methyl protons of isobutyramide (Figure 5). The assignments of the resonances of the carbon-bound protons in the nmr spectrum of NADH (Oppenheimer *et al.*, 1971; Jardetzky and Wade-Jardetzky, 1966) and isobutyramide (Mildvan and Weiner, 1969a) have previously been described, and the phosphorus resonances were observed at 9.1 ppm upfield from H_3PO_4 (64%) in D_2O. The diamagnetic corrections to the relaxation rates were made as described in the Experimental Section.

For each observed nucleus of NADH and of isobutyramide, the paramagnetic effect of EI· on the transverse relaxation rate ($1/fT_{2p}$) was at least an order of magnitude greater than its effect on the longitudinal relaxation rate ($1/fT_{1p}$) as shown in Table II. Moreover the relaxation rates of the protons were significantly less at 220 MHz than at 100 MHz (Table II). As discussed in detail elsewhere (Nowak and Mildvan, 1972) these findings indicate that $1/fT_{1p}$ and $1/fT_{2p}$ are not limited by chemical exchange since the largest value of $1/fT_{2p}$ for

TABLE III: Correlation Times (τ_c), Correlation Functions ($f(\tau_c)$), and Distance Parameters for the Binary and Ternary Complexes of Spin-Labeled Alcohol Dehydrogenase.[a]

Method[b]	Parameter	Nucleus							
		H_8A	H_2A	H_2P	H'A	H_6P	H_4P	IBA	Av[c]
Binary complex									
T_1/T_2 (100 MHz)	τ_c ($\times 10^{-9}$ sec)	6.3	13.5	6.5	10.4	9.2	5.1		8.5
	$f(\tau_c)$	1.2	0.5	1.1	0.7	0.8	1.4		1.0
T_1/T_2(220 MHz)[d]	τ_c	<7.2	4.2	6.8	<7.2	2.2	7.2		5.1
	$f(\tau_c)$	>1.0	1.6	1.1	>1.0	2.3	1.0		1.5
T_1(100)/T_1(220)	τ_c	3.5	0.8	2.1	5.1	0.9	3.0		2.6
	$f(\tau_c)$	1.8	1.9	2.3	1.4	2.0	2.0		1.9
	r(Å)	11.8	13.4	10.8	11.6	9.3	8.6		
Ternary complex									
T_1/T_2(100 MHz)	τ_c	4.8	14.6	5.4	19.4	15.7	7.3	15.7	11.7
	$f(\tau_c)$	1.4	0.5	1.3	0.4	0.5	1.0	0.5	0.8
T_1/T_2(220 MHz)[d]	τ_c	<7.2	<7.2	4.1	<7.2	2.6	2.5	6.3	3.9
	$f(\tau_c)$	>1.0	>1.0	1.6	>1.0	2.1	2.2	1.1	1.8
T_1(100)/T_1(220)	τ_c	>1.0	1.3	2.1	>1.0	1.1	1.5	1.9	1.6
	$f(\tau_c)$	>2.0	2.3	2.3	>2.0	2.2	2.4	2.3	2.3
	r(Å)	11.1	12.8	10.1	12.9	9.2	8.9	11.9	

[a] $f(\tau_c)$ calculated for 100 MHz. [b] From measurements of the line width at half-height of the epr spectra, estimates of τ_c were 16.1×10^{-9} and 17.5×10^{-9} sec for the binary and ternary complexes, respectively. The estimates of $f(\tau_c)$ were 0.5×10^{-9} and 0.4×10^{-9}. [c] Upper or lower limit values were not used in calculating the average. [d] T_2 data at 220 MHz taken on samples containing 13% of the labeled aggregate described in the text.

NADH (1200 sec^{-1}) and for isobutyramide (230 sec^{-1}) sets lower limits on the respective exchange rate of these substrates from the ternary complex. That this maximal $1/fT_{2p}$ value for NADH is in the fast exchange case (Luz and Meiboom, 1964), and provides a lower limit to the exchange rate, is established by its negative temperature dependence (E_{act} = 5.0 ± 2.4 kcal/mol) between 1 and 30°, determined under the same conditions as described in Figure 5. Hence the values of $1/fT_{1p}$, which are smaller, may be used to determine distances between the unpaired electron of the spin-label and the protons of the bound coenzyme or substrate analog using the dipolar term of the Solomon–Bloembergen equation as described in the Experimental Section. As discussed in detail elsewhere (Mildvan and Engle, 1972; Fung *et al.*, 1973; Nowak *et al.*, 1973) the precise determination of distances requires an independent evaluation of the correlation function, $f(\tau_c)$, for the electron–proton dipolar interaction. In the present case, $f(\tau_c)$ was determined by three independent nmr methods as shown in Table III. Moreover, the τ_c values determined by the nmr methods are the same order of magnitude as the electron spin relaxation estimated from the epr line width (~16 nsec), and also, the overall shape and width of the epr spectra of the bound spin-label (Figure 2) are very similar to calculated spectra assuming a rotational correlation time for the radical of ~20 nsec (McCalley *et al.*, 1972). Although the average values of τ_c determined by the various methods differed by factors of 5.7 and 7.3 for the binary and ternary complexes, respectively, the corresponding values of $f(\tau_c)$ differed only by factors of 1.9 and 2.9 (Table III). The $f(\tau_c)$ values based on the most extensive data (and showing the highest precision of ±28 and ±40% for the binary and ternary complexes) were calculated from the T_{1p}/T_{2p} ratios at 100 MHz taken on identical samples at about the same time. The average of these values was used for the distance determinations (Table III) introducing an average uncertainty of 8% in the absolute r values. If the average $f(\tau_c)$ values from all of the nmr methods were used, together with the observed maximal variation in $1/fT_{1p}$, the maximal error in the absolute r values would be 10% for the binary complex and 13% for the ternary complex.

Due to an uncertainty of ±10% in $1/T_{1p}$ and because of the sixth root relationship between distance and relaxation (Mildvan and Engle, 1972), the relative r values shown in Table III have a calculated uncertainty of 1.6%. Because of the possible variability in τ_c (Table III), suggested by the high T_{1p}(100 MHz)/T_{1p}(220 MHz) for H_8Ad (Table II), and our neglect of small effects due to internal rotation, the error in

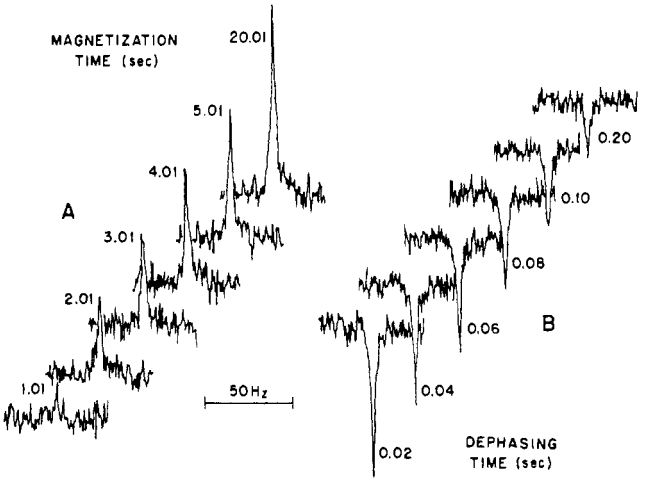

FIGURE 6: Magnetic relaxation of the pyrophosphate phosphorus of NADH (15 mM) in the presence of EI · (1200 μM in enzyme-bound spins) and isobutyramide (10 mM). (A) T_1 measurement by the partially relaxed Fourier transform method of McDonald and Leigh (1973), (B) T_2 measurement by the pulsed Fourier transform method. Conditions: 50% D_2O (pH 7.5), 23 ± 1°. Initial sample volume was 1.2 ml.

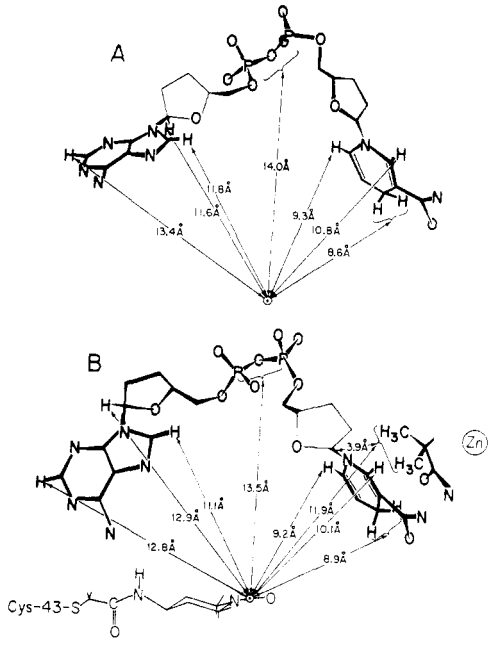

FIGURE 7: Geometry of the binary EI·NADH (A) and ternary EI·NADH–isobutyramide complexes (B) consistent with the distances between the unpaired electron of the spin-label and the protons and phosphorus of bound NADH and isobutyramide. The distance between NADH and isobutyramide is from Mildvan and Weiner (1969a). The location of the Zn and cysteine-43 is from the crystallographic model of the ternary liver alcohol dehydrogenase–ADP-ribose–*o*-phenanthroline complex (Branden *et al.*, 1973), and the amino acid sequence of the yeast enzyme (Jornvall, 1973).

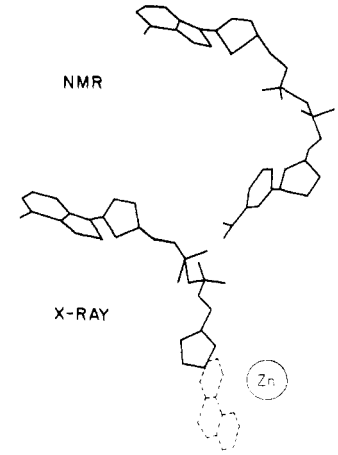

FIGURE 8: Comparison of the conformation of NADH bound to spin-labeled yeast alcohol dehydrogenase from nmr data in solution with that of ADP-ribose bound to liver alcohol dehydrogenase from crystallographic data (Branden *et al.*, 1973).

the relative distances introduced by assuming a constant τ_c may approach 10% in certain cases.

A displacement experiment indicated negligible outer sphere contributions to $1/fT_{1p}$ and $1/fT_{2p}$, within their respective experimental errors. Lyophilized NAD^+ was added to a solution containing spin-labeled enzyme (1.38 mM), NADH (14.7 mM), and isobutyramide (5.0 mM), in an amount (496 mM) sufficient to displace 68% of the NADH from the spin-labeled enzyme, as estimated using the dissociation constants of Table I. Under these conditions, the $1/fT_{1p}$ of the C_4 protons of NADH decreased by 66% and $1/fT_{2p}$ decreased by 80%. All of the other resonances of NADH were obscured by the excess NAD^+. Hence no additional errors are introduced into the distance calculations by neglecting outer sphere relaxation. The distances were used to construct the models shown in Figure 7. These models may represent the average of several structures which interconvert rapidly on an nmr time scale which is $>10^3$ sec^{-1} from the data of Table II.

Description of the Nmr Model of Bound NADH. The spin–proton distances in the binary EI·NADH (Figure 7A) and ternary EI·NADH–isobutyramide complexes (Figure 7B) require the adenine ring to be in the anti conformation with respect to the ribose ring in agreement with the crystal structure of several dinucleotides (Kim *et al.*, 1973, and Sussman *et al.*, 1972) and with that of NAD^+ bound to lactate dehydrogenase (Chandrasekhar *et al.*, 1973) and malic dehydrogenase (Webb *et al.*, 1973). Similarly, the distances require an open conformation for bound NADH since a closed or stacked conformation would force the adenine and dihydropyridine rings to approach each other much more closely (~1 Å) than the allowed stacking distance of 3.5 Å. An open conformation is also required by our detection of fluorescence enhancement yet our failure to detect fluorescence transfer from adenine to dihydropyridine and by our failure to observe an upfield chemical shift of the carbon bound protons of the dihydropyridine ring in these complexes in agreement with the nmr data of Lee *et al.* (1973). The distances are consistent with, but do not require, the more stable anti conformation for the dihydropyridine ring with respect to the ribose ring. These considerations, together with the known conformations of ribose phosphate, and a staggered conformation of the pyrophosphate chain have been incorporated into the structure of the models.

A comparison of the models of the binary and ternary complexes (Figures 7A and 7B) suggests that the binding of isobutyramide to EI·NADH has caused a conformation change in the bound coenzyme or, alternatively, that the spin-label has moved. Upon binding of isobutyramide, the adenine ring has rotated toward the unpaired electron by ~1 Å and the dihydropyridine ring has moved away from the spin by ~0.5 Å. The dihedral angle between the adenine and dihydropyridine rings (~90°, Figure 7) may alter slightly on forming the ternary complex. Analogous changes have recently been found by crystallographic studies of lactate dehydrogenase (Chandrasekhar *et al.*, 1973).

The location of the bound substrate analog isobutyramide with respect to the bound NADH is not uniquely determined by the single calculated distance (11.9 Å) from the spin-label. However, using the nicotinamide–ribose bond as a second reference point and the previously estimated distance of 3.9 Å between the methyl protons of isobutyramide and an unpaired electron at this point on liver alcohol dehydrogenase (Mildvan and Weiner, 1969a) a unique location for the bound analog is obtained. While equating the isobutyramide–nicotinamide distances on yeast and liver alcohol dehydrogenases is speculative, it is of interest that the position of isobutyramide so determined places it in molecular contact with the face of the dihydropyridine ring in a proper orientation to accept a hydride ion.

Comparison of Nmr Model with Crystallographic Models. A comparison of the model of the ternary complex from the nmr data (Figure 8) with the crystallographic model of the ternary liver alcohol dehydrogenase–ADP-ribose–*o*-phenanthroline complex (Branden *et al.*, 1973), with the help of Dr. Carl Branden and his colleagues, indicates that the conformation of NADH on yeast alcohol dehydrogenase in solution is very similar to that of ADP-ribose on liver alcohol

dehydrogenase in the crystalline state.[2] The position of the unpaired electron of the nitroxide radical would be 8–9 Å from the crystallographically located sulfur of cysteine-43 (see Figure 7B), and a groove exists in the protein structure in which the extended spin-label could lie (Branden *et al.*, 1973). A comparison of the nmr model of the ternary complex, which locates the dihydropyridine ring and the substrate analog, with the crystallographic model, which locates the Zn atom (Figure 7B), indicates that the dihydropyridine ring is 6–7 Å from the Zn and that the substrate analog lies between the dihydropyridine ring and the Zn atom at a position 2–3 Å from the metal which is appropriate for direct coordination. Coordination of the oxygen of an aldehyde substrate by the enzyme-bound Zn would polarize the carbonyl group, as required by a study of substituent and isotope effects on the yeast alcohol dehydrogenase reaction (Klinman, 1972), and would facilitate hydride transfer, as established by a highly appropriate model reaction (Creighton and Sigman, 1971).

Coordination to Zn was previously suggested as an explanation for the low rate of binding of ethanol to liver alcohol dehydrogenase (Mildvan and Weiner, 1969a; Mildvan *et al.*, 1972).

From the quenching of fluorescence of NADH bound to Co^{2+}-substituted liver alcohol dehydrogenase, Takahashi and Harvey (1973) have estimated dihydropyridine to Co^{2+} distances of 19 and 23 Å in the binary and ternary isobutyramide complexes, respectively. However, these distances are arbitrary since they were calculated using an assumed orientation factor of $^2/_3$, the value of which could lie between 0 and 4 (Takahashi and Harvey, 1973). An orientation factor of 7×10^{-4}, which cannot be excluded, would lead to a distance of ~6 Å in the binary complex and ~7 Å in the ternary complex, in accord with our model.

As shown in Figure 9, the nmr model of the binary EI· NADH complex is similar to those proposed for NAD^+ bound to lactate dehydrogenase (Chandrasekhar *et al.*, 1973) and malic dehydrogenase (Webb *et al.*, 1973), in that the pyridine and adenine rings are approximately perpendicular. However, differences are noted in the dihedral angle between the ribose ring oxygen and the C_6 position of the pyridine ring (χ_N as defined by Chandrasekhar *et al.*, 1973). The value of χ_N for NADH on alcohol dehydrogenase from our nmr model is ~30°, assuming the isobutyramide to be on the A face of the dihydropyridine ring, while the values of χ_N for NAD^+ are 90 and 130° on lactate dehydrogenase (Chandrasekhar *et al.*, 1972) and malic dehydrogenase (Webb *et al.*, 1973), respectively. These differences could result from differences in the dehydrogenase sites or from a difference in the preferred conformation of the reduced coenzyme. As pointed out by Velick (1961), conformational changes may occur, upon reduction of an aromatic pyridinium ribonucleoside, altering the relative positions of the ribose and pyridine rings. Analogous conformational differences between oxidized and reduced coenzymes on alcohol dehydrogenase are possible in view of the order of magnitude difference in binding constants for both the active (Sund and Theorell, 1963) and modified (Figure 3) enzymes.

Finally, since all components bind to EI· with the correct affinity (Table I) and with a reasonable geometry (Figures 7 and 8) it is not obvious why EI· is inactive. Clearly something must be wrong with its structure. While it is at present

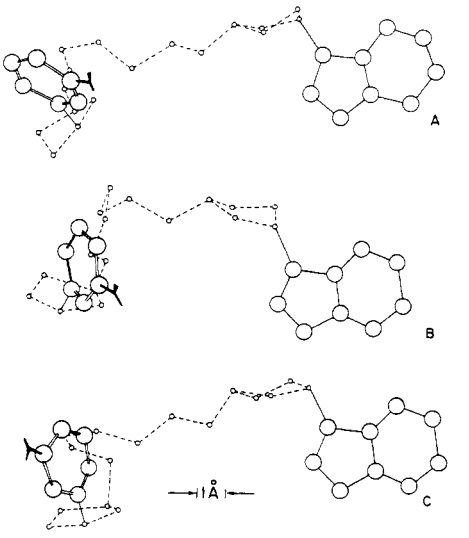

FIGURE 9: Comparison of conformations of NAD^+, as determined crystallographically, to that of the NADH conformation from nmr data: (A) conformation of NAD^+ bound to lactate dehydrogenase (binary complex, taken from Chandrasekhar *et al.*, 1973), (B) approximation of the conformation of NAD^+ in binary complex with malic dehydrogenase (produced from the interatomic distances and torsion angles of Webb *et al.*, 1973), (C) conformation of NADH bound to EI·. For simplicity, only the backbone of each dinucleotide has been included.

unclear from our work what is wrong, recent observations by Sanderson and Weiner (1973) and by Dickinson (1972) provide clues. Thus, unlike the native enzyme, the iodoacetamide inactivated enzyme shows no increase in the intensity or polarization of fluorescence of bound NADH by substrates (Sanderson and Weiner, 1973) and no intensification of the NADH fluorescence by acetamide (Dickinson, 1972). Since the present work indicates that ethanol, isobutyramide, and acetaldehyde can still bind to inactive alcohol dehydrogenase, the precise orientation of the bound substrate with respect to the dihydropyridine ring of NADH may be altered in the modified enzyme. Hence, we cannot state with certainty that the structural juxtaposition of the coenzyme and substrate indicated by this study is that of the active alcohol dehydrogenase complex. We are presently trying to isolate an active alcohol dehydrogenase from yeast grown in a cobalt-rich medium. A comparison of a similar set of experiments, using the metal as paramagnetic reference point, to those presented here should unambiguously position the coenzyme and substrate.

Acknowledgments

We are grateful to Dr. Carl Branden, Dr. Lawrence Webb, Dr. Leonard Banaszak, and Dr. Helen Berman for advice in the construction of the molecular models, to Dr. Judith P. Klinman for valuable discussions, and to Mr. Ronald Abramson for help with the nmr measurements.

References

Boyer, P. D., and Theorell, H. (1956), *Acta Chem. Scand. 10*, 447.

Branden, C. I., Eklund, H., Nordstrom, B., Boiwe, T., Soderlund, G., Zeppezauer, E., Ohlsson, I., and Åkeson, A. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 2439.

Carr, H. Y., and Purcell, E. M. (1954), *Phys. Rev. 94*, 630.

[2] The conformations of the pyrophosphate region of NADH in the nmr model and of ADP-ribose in the crystallographic model are not precisely defined.

Chandrasekhar, K., McPherson, A., Jr., Adams, M. J., and Rossman, M. G. (1973), *J. Mol. Biol. 76*, 503.
Creighton, D. J., and Sigman, D. S. (1971), *J. Amer. Chem. Soc. 93*, 6314.
Dickinson, F. M. (1970), *Biochem. J. 120*, 821.
Dickinson, F. M. (1972), *Biochem. J. 126*, 133.
Fung, C. H., Mildvan, A. S., Allerhand, A., Komoroski, R., and Scrutton, M. C. (1973), *Biochemistry 12*, 620.
Harris, J. I. (1964), *in* Structure and Activity of Enzymes, Goodwin, T. W., Harris, J. I., and Hartley, B. S., Ed., London, Academic Press, p 97.
Hayes, J. E., Jr., and Velick, S. F. (1954), *J. Biol. Chem. 207*, 225.
Jardetzky, O. (1964), *Advan. Chem. Phys. 7*, 499.
Jardetzky, O., and Wade-Jardetzky, N. G. (1966), *J. Biol. Chem. 241*, 85.
Jornvall, H. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 2295.
Kim, S. H., Berman, H. M., Seeman, N. C., and Newton, M. D. (1973), *Acta Crystallogr., Sect. B 29*, 703.
Klinman, J. P. (1972), *J. Biol. Chem. 247*, 7977.
Lee, C., Eichner, R. D., and Kaplan, N. O. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 1593.
Luz, Z., and Meiboom, S. (1964), *J. Chem. Phys. 40*, 2686.
McCalley, R. C., Shimshick, E. J., and McConnell, H. M. (1972), *Chem. Phys. Lett. 13*, 115.
McDonald, G. G., and Leigh, J. S., Jr. (1973), *J. Magn. Resonance 9*, 358.
Mildvan, A. S., and Engle, J. (1972), *Methods Enzymol. 26*, 654.
Mildvan, A. S., Waber, L., Villafranca, J. J., and Weiner, H. (1972), *in* Structure and Function of Oxidation Reduction Enzymes, Akeson, A., and Ehrenberg, A., Ed., Oxford, Pergamon Press, p 745.
Mildvan, A. S., and Weiner, H. (1969a), *J. Biol. Chem. 244*, 2465.
Mildvan, A. S., and Weiner, H. (1969b), *Biochemistry 8*, 552.
Nowak, T., and Mildvan, A. S. (1972), *Biochemistry 11*, 2819.
Nowak, T., Mildvan, A. S., and Kenyon, G. L. (1973), *Biochemistry 12*, 1690.
Nygaard, A. P., and Theorell, H. (1955), *Acta Chem. Scand. 9*, 1300.
Oppenheimer, N. J., Arnold, L. J., and Kaplan, N. O. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 3200.
Phillips, D. C. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 484.
Sanderson, J. A., and Weiner, H. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 532.
Sloan, D. L., Jr., and Mildvan, A. S. (1973), Proceedings of the International Symposium on Alcohol and Aldehyde Metabolizing Systems, Truman, R., Ed. New York, N. Y., Academic Press (in press).
Spallholz, J. E., and Piette, L. H. (1972), *Arch. Biochem. Biophys. 48*, 596.
Sund, H., and Theorell, H. (1963), *Enzymes, 2nd Ed. 7*, 25.
Sussman, J. L., Seeman, N. C., Kim, S. H., and Berman, H. M. (1972), *J. Mol. Biol. 66*, 403.
Takahashi, M., and Harvey, R. A. (1973), *Biochemistry 12*, 4743.
Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 15*, 1127.
Twu, J., Chin, C. C. Q., and Wold, F. (1973), *Biochemistry 12*, 2856.
Twu, J., and Wold, F. (1973), *Biochemistry 12*, 381.
Vallee, B. L., and Hoch, F. L. (1955), *Proc. Nat. Acad. Sci. U. S. 41*, 327.
Velick, S. F. (1958), *J. Biol. Chem. 233*, 1455.
Velick, S. F. (1961), *in* Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., The Johns Hopkins Press, p 108.
Webb, L. E., Hill, E. J., and Banaszak, L. J. (1973), *Biochemistry 12*, 5101.
Wien, R. W., Morrisett, S. D., and McConnell, H. M. (1972), *Biochemistry 11*, 3707.
Winer, A. D., and Theorell, H. (1960), *Acta Chem. Scand. 14*, 1729.