^{31}P Nuclear Magnetic Resonance Studies of the Interaction of Pyridine Nucleotide Coenzymes with Dehydrogenases†

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ABSTRACT: ^{31}P nuclear magnetic resonance spectra of the pyrophosphate group in NAD^+ and NADH were recorded in the presence of beef heart lactate dehydrogenase and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. At high lactate dehydrogenase concentrations (60 mg/ml), two NADH resonances are observed: a slowly exchanging peak which is shifted 1.9 ppm downfield (relative to free NADH) and a rapidly exchanging peak with a downfield shift of 0.5–0.6 ppm. At lower concentrations (15 mg/ml) only the rapidly exchanging peak is observed thus indicating that the peak observed at −1.9 ppm is due to coenzyme bound to an aggregated enzyme species. With NAD^+, rapid exchange and downfield shifts are observed at both enzyme and concentrations, with shifts of about 1.5 ppm and 0.6 ppm at 60 and 15 mg/ml, respectively. In the presence of glyceraldehydephosphate dehydrogenase, the results are independent of enzyme concentration, and slow exchange and upfield shifts of 0.4–0.6 ppm occur with each coenzyme. These data indicate that the environment of the pyrophosphate group of oxidized and reduced coenzyme is the same for a given dehydrogenase, but is different in one enzyme from the other. The resonances observed with glyceraldehydephosphate dehydrogenase are broader than those observed with lactate dehydrogenase. This is indicative of either shorter relaxation times with the former enzyme, or the presence of multiple, unresolved resonances.

During the past several years, there have been many studies in which nuclear magnetic resonance (NMR) has been used to monitor the interaction between various dehydrogenases and their pyridine nucleotide coenzymes, NAD^+ and NADH (Jardetzky et al., 1963; Czeisler and Hollis, 1973; Lee et al., 1973; Sloan and Mildvan, 1974, and other references in these articles). In most of these studies, the coenzyme is present in excess over the enzyme, and proton resonances of the nicotinamide and/or adenine moieties of the coenzymes are observed at different coenzyme/enzyme ratios. From the changes caused by the enzyme in the chemical shifts and line widths of the coenzymes' resonances, information on the conformation of the bound coenzyme, as well as on the kinetics of binding and unbinding has been obtained. Also, relaxation effects due to paramagnetic metals and spin labels have been used to delineate various distance relationships among enzyme, coenzyme, and substrate.

In the present study the binding of NAD^+ and NADH to two dehydrogenases, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and beef heart lactate dehydrogenase, has been studied using ^{31}P NMR. X-Ray structures of these two enzymes have been recently obtained (Adams et al., 1973; Buehner et al., 1973) and in both proteins it was observed that the pyrophosphate group was intimately involved in coenzyme binding. It was therefore believed that a probe of this region would yield information not obtainable through monitoring the nicotinamide or adenine rings. Also, in ^{31}P studies, unlike in proton work, it is not necessary to have an excess of coenzyme present, since there are no interfering ^{31}P signals due to the enzyme. This means that systems in which the coenzyme exchange rate between the enzyme and bulk solution is slow, i.e., $k_{off} < 2\pi\Delta$, where Δ is the chemical shift difference between free and bound forms, can be studied. This slow exchange rate is usually present whenever very strong binding occurs, such as in the glyceraldehydephosphate dehydrogenase–NAD^+ system. In proton NMR work, due to the large number of enzyme protons, an excess of coenzyme must be added if coenzyme resonances are to be observed, and the coenzyme exchange rate must be relatively fast on the NMR time scale ($k_{off} \geq 2\pi\Delta$) for useful information on the chemical shift of the bound coenzyme to be obtained.

The ^{31}P results presented here allow comparisons to be made between the NAD^+ binding sites of two different dehydrogenases, as well as comparing the binding of oxidized and reduced coenzyme to the same enzymes. Also, evidence for aggregation of lactate dehydrogenase at high concentrations is presented.

Experimental Section

Materials. Lactate dehydrogenase (beef heart, type III) was purchased from Sigma and the H_4 isozyme prepared from the commercial material according to Pesce et al. (1964).. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was isolated according to Bloch et al. (1971). Coenzymes were purchased from Sigma. When NADH of higher purity was desired, the material from Sigma was purified according to Stinson and Holbrook (1973).

Preparation of Enzyme Samples. The crystalline suspension of lactate dehydrogenase was centrifuged and the precipitate dissolved in 5 m*M* EDTA–0.05 *M* Tris (pH 7.0) and dialyzed against the same buffer. The enzyme solution was then dialyzed against a buffer of the same composition made up in D_2O (the pD was taken to be the meter reading +0.4) and concentrated by vacuum dialysis to about 60 mg/ml. Glyceraldehydephosphate dehydrogenase solutions

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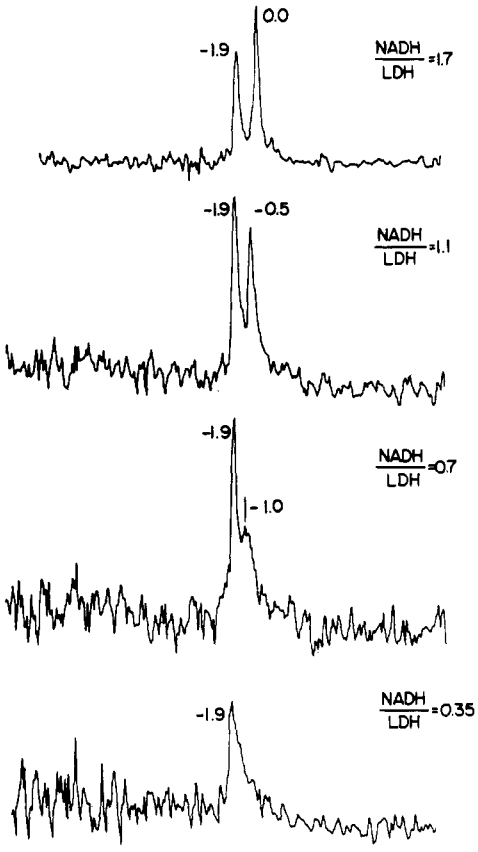

FIGURE 1: ^{31}P spectra of NADH at varying concentrations in the presence of lactate dehydrogenase (LDH) (50 mg/ml) in 0.05 *M* Tris, 5 m*M* EDTA, and D_2O, "pD" 7.0. The chemical shifts in ppm relative to the ^{31}P resonance of free NADH are shown next to the peaks. Negative numbers signify downfield shifts. The ratio of coenzyme to enzyme is the number of moles of NADH added per mole of enzyme subunit. The ratio therefore refers to initial, rather than free concentrations.

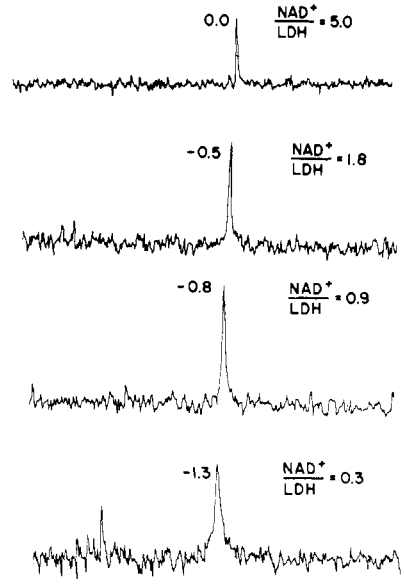

FIGURE 2: ^{31}P spectra of NAD^+ in the presence of lactate dehydrogenase (50 mg/ml). See Figure 1 for conditions.

were prepared in the same manner, except the buffer contained 5 m*M* EDTA–0.1 *M* KCl (pH (or pD) 7.5). Coenzyme solutions were made up to concentrations of about 15 mg/ml and the desired amount was added volumetrically to the enzyme solution. The exact concentrations of all coenzyme and enzyme solutions were determined spectrophotometrically, using known extinction coefficients and a Cary 15 spectrophotometer.

Spectra were obtained on a Varian XL-100 spectrometer interfaced to a Bruker 1080 computer, operating in the Fourier transform mode at 40.5 MHz. The instrument was locked on the D_2O of the enzyme solutions. Proton decoupling was employed and the temperature was 33 ± 2°C. Spectra were obtained using a spectral width of 2500 Hz, 4K of memory (resolution = 1.2 Hz), and repetition rates of from 1.3 to 2.0 sec. Total accumulation time varied from 1 to 24 hr, depending on the coenzyme concentration of the sample. Since varying accumulation times were used absolute intensity comparisons cannot be made between different spectra.

Peak positions were determined from the display on the computer oscilloscope by reading the address of the cursor when it was set at the top of each peak. The D_2O lock served as internal standard and the shifts are referenced to the position of the ^{31}P resonance of NADH in the absence of enzyme. Upfield shifts are given positive values. To check for an enzyme-induced shift of the D_2O signals, and/or susceptibility effects at high enzyme concentration, spectra were taken in which the coenzyme to enzyme ratio was very high (>10) and the spectra compared to those obtained in the absence of coenzyme. At high coenzyme/enzyme ratios, specific enzyme induced effects on the coenzyme resonances would be very small, so any difference observed in the presence or absence of enzyme would be due to nonspecific effects. Such effects were found to be under 0.1 ppm at the highest coenzyme concentrations employed (and proportionately less at lower concentrations). Shifts with lactate dehydrogenase are reproducible to ±0.1 ppm. Due to the diffuse nature of the resonances obtained with glyceraldehydephosphate dehydrogenase, the uncertainty in peak position is 0.2 ppm.

Results

In Figure 1 are shown ^{31}P spectra of NADH in the presence of lactate dehydrogenase (Sigma, from beef heart, type III). All of the spectra exhibit a peak which is 1.9 ppm downfield of the position of the free NADH resonance. As the NADH concentration is raised, another peak is observed, which shifts upfield with increasing NADH concentration until it reaches the position of free NADH. The association constant between lactate dehydrogenase and NADH is about 10^7 M^{-1} (Anderson and Weber, 1965). Therefore, at coenzyme/enzyme ratios of less than unity, the observed resonances are due solely to bound coenzyme, while at higher NADH concentration, there is a contribution due to free coenzyme.

Since the observation of two peaks was somewhat unexpected, efforts were made to determine if enzyme heterogeneity or impure coenzyme could be contributing to the observed spectral pattern. It was found that studies with the purified H_4 isozyme of lactate dehydrogenase, or with a rechromatographed NADH, yielded spectra identical with those shown in Figure 1.

Addition of varying amounts of NAD^+ to lactate dehydrogenase results in ^{31}P spectra (Figure 2) in which only one peak is present at all coenzyme concentrations. At subsaturating levels of coenzyme, this resonance has a chemical shift which is similar to that of the NADH resonance shown in Figure 1 (bottom spectrum). Addition of excess NAD^+ causes the ^{31}P peak to move gradually upfield until it as-

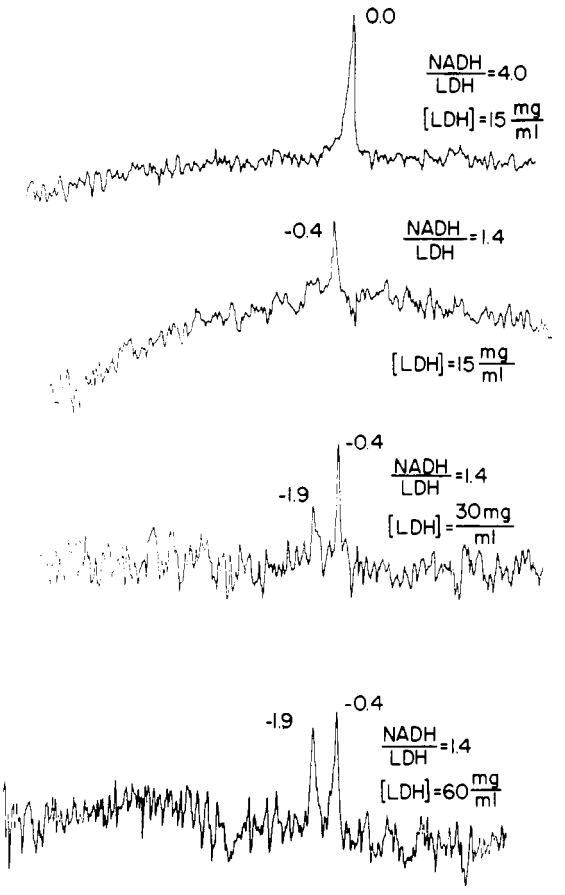

FIGURE 3: ^{31}P spectra of NADH in the presence of various concentrations of lactate dehydrogenase.

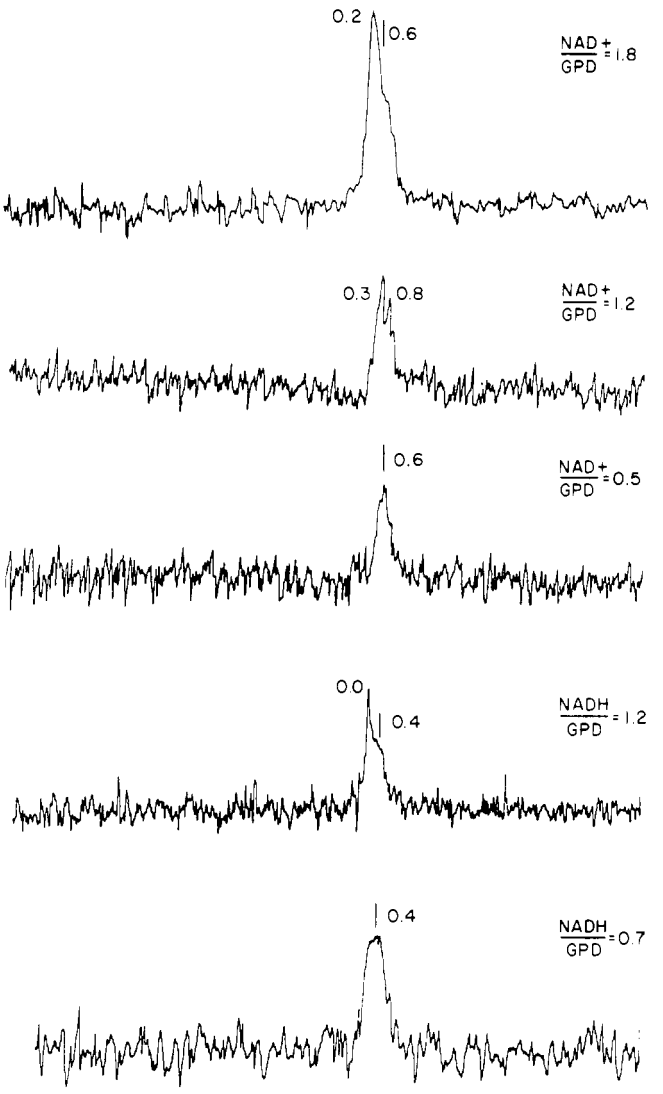

FIGURE 4: ^{31}P spectra of NADH and NAD^+ in the presence of glyceraldehyde-3-phosphate dehydrogenase (50 mg/ml) in 0.1 *M* KCl, 5 m*M* EDTA, and D_2O, "pD" 7.5. As in Figure 1, positions of peaks are relative to the position of the free NADH resonances.

sumes a position close to that of the free NAD^+ peak when a large excess of NAD^+ is present. The binding of NAD^+ to lactate dehydrogenase is characterized by an association constant of 10^3–10^4 M^{-1} (Stinson and Holbrook, 1973). Therefore, in the concentration range of these studies, there is always a significant amount of free NAD^+ present. Thus, all spectra of NAD^+ in the presence of lactate dehydrogenase include a contribution due to free NAD^+, and a completely bound NAD^+ cannot be observed.

When a solution containing 1:1.4 lactate dehydrogenase/NADH was diluted, the results shown in Figure 3 were obtained. The downfield peak diminishes in relative intensity on going from 60 to 30 mg/ml and virtually disappears in going to 15 mg/ml. Addition of excess NADH to the most dilute solution (top spectrum, Figure 3) causes the one observed peak to move to the position of the free coenzyme resonance. The upfield resonance observed in the spectra in Figure 3 is an average of free and bound resonances. The chemical shift of the bound form can be determined from the spectrum second from the top in Figure 3 from the relation

$$\Delta_{obsd} = \Delta_{bound} f_{bound}$$

where Δ_{obsd} and Δ_{bound} refer to the observed and bound chemical shifts, and f_{bound} is fraction of coenzyme which is bound. Since the enzyme is saturated at these conditions $f = 1/1.4$ and Δ_{bound} is therefore equal to -0.4×1.4 or about -0.6 ppm. In the bottom two spectra of Figure 3, coenzyme and enzyme which give rise to the slowly exchanging peak do not contribute to the shift of the rapidly exchanging one. Thus in calculating the expected shift of the upfield peak, the coenzyme and enzyme concentrations are both lowered, but their ratio remains between 0.5 and 0.7 (f_{bound} is between 2.0 and 1.4), and the observed shift of -0.4 ppm is therefore within experimental error of the calculated value.

On addition of NAD^+ to a dilute solution of lactate dehydrogenase, downfield shifts are observed, though these are smaller in magnitude than those observed with 60-mg/ml solutions. As is the case with NADH, the position of the bound NAD^+ resonance is -0.5 to -0.6 ppm.

Spectra of NADH and NAD^+ in the presence of glyceraldehyde-3-phosphate dehydrogenase are shown in Figure 4. With the reduced coenzyme, a broad resonance, centered at 0.4 ppm, is observed. When an excess of coenzyme is added, the broad resonance is still present, in addition to a sharp peak due to free coenzyme, thus indicating slow exchange between free and bound coenzyme. With the oxidized coenzyme, similar results are obtained, as there is a 0.4–0.6 ppm upfield shift relative to the free NAD^+ resonance. Since free NAD^+ is 0.2 ppm upfield of free NADH (Blumenstein and Raftery, 1972), the position of the bound NAD^+ is 0.6–0.8 on the scale used in this paper. The spec-

tra with both reduced and oxidized coenzymes are broader than those spectra observed with lactate dehydrogenase. Spectra obtained at 20 mg/ml of glyceraldehydephosphate dehydrogenase are identical with those seen at an enzyme concentration of 60 mg/ml.

Spectra of NAD^+ and NADH in the absence of enzymes were recorded at various concentrations. For both coenzymes, the chemical shift position was essentially unchanged (i.e., shift ≤ 0.05 ppm) in going from 2×10^{-4} to 3×10^{-2} *M*.

Discussion

The ^{31}P results presented in this paper appear to indicate two classes of NADH binding sites on beef heart lactate dehydrogenase: a strong site, from which slow exchange between bound and unbound NADH is observed, and a weaker site, from which rapid exchange occurs. This result was surprising, since previous binding studies (Anderson and Weber, 1965) have shown only one class of site. This seeming contradiction was resolved by observing the dependence on enzyme concentration of the ^{31}P spectra, while keeping the coenzyme/enzyme ratio constant. It was seen that the low-field resonance, which displayed slow exchange, disappeared on lowering the enzyme concentration from 60 to 15 mg/ml, leaving only the high field, fast exchange peak. Thus, it is highly probable that the downfield resonance observed at high enzyme concentration was due to coenzyme which was binding to an aggregated form of lactate dehydrogenase.[1]

A proton NMR study of the interaction between chicken M_4 lactate dehydrogenase and NADH (Sarma and Kaplan, 1970) was interpreted as indicating fast exchange between enzyme and coenzyme, but a later communication from the same laboratory (Lee *et al.*, 1973) reversed this conclusion and determined slow exchange to be present. The proton work was performed at enzyme concentrations of about 60 mg/ml, so aggregation may have been a factor which complicated these experiments, though the fact that the H_4 isozyme of lactate dehydrogenase (used in the present ^{31}P work) tends to aggregate, does not necessarily mean that the M_4 isozyme also does.

The present results point out the necessity of checking the effect of enzyme concentration on NMR results, since with the high concentration generally employed in NMR experiments, aggregation may often be a problem.

Differences between Lactate Dehydrogenase and Glyceraldehyde-3-phosphate Dehydrogenase. The crystal structure of dogfish M_4 lactate dehydrogenase (Adams et al., 1973) indicates that the pyrophosphate of NAD^+ interacts with an arginine residue, while crystallographic data on lobster glyceraldehyde-3-phosphate dehydrogenase (Buehner et al., 1973) show binding of the pyrophosphate to a lysine. If one assumes the structures of the enzyme species employed here (beef heart H_4 lactate dehydrogenase and rabbit muscle glyceraldehydephosphate dehydrogenase) to be similar to those on which X-ray work was done, the observation of ^{31}P chemical shifts different magnitudes is not too surprising. What is somewhat unexpected, since both lysine and arginine are positively charged, is the different direction of the shifts, with downfield shifts observed for lactate dehydrogenase (at all concentrations) and upfield shifts seen with glyceraldehydephosphate dehydrogenase. We have no explanation for the opposite directions of the observed shifts, but it may indicate an effect other than a simple charge interaction between the negatively charged pyrophosphate and a positively charged amino acid. A distorted phosphorus–oxygen bond, for example, or an effect due to the dielectric constant of the coenzyme environment, would contribute to the observed shifts. Another possible cause for the downfield shift observed with lactate dehydrogenase is that the delocalized guanidinium cation of arginine gives rise to an electronic effect similar to the ring current of a benzene ring. If the ^{31}P nuclei are in the same plane as the charge, they will therefore undergo a downfield shift.

Resonances observed with glyceraldehydephosphate dehydrogenase were consistently broader than those seen with lactate dehydrogenase (30–40 Hz wide as opposed to 15–20 Hz). This broadening indicates either that the T_2 values in the presence of the former enzyme are shorter than in the presence of the latter, or that there are several overlapping peaks present with glyceraldehydephosphate dehydrogenase. The proper choice between these possibilities cannot easily be made at 40 MHz alone, due to the fact that any chemical shift differences which might be present would be small relative to the line widths, but studies at higher frequencies, utilizing superconducting spectrometers, should be very helpful in resolving the question.

Comparison of Reduced and Oxidized Coenzymes. For each dehydrogenase, the chemical shifts and line shapes were very similar for reduced and oxidized coenzymes. At high lactate dehydrogenase concentrations, the slowly exchanging peak observed with NADH had a somewhat greater downfield shift than did the NAD^+ resonance, but the latter was presumably an average peak due to NAD^+ bound to aggregated enzyme, as well as NAD^+ bound to the normal tetrameric enzyme, and this averaged shift would be smaller than the shift of NADH, which was solely due to the aggregated protein. At lower enzyme concentrations, the bound shifts of reduced and oxidized coenzymes were very similar, as was the case in the presence of glyceraldehydephosphate dehydrogenase. The two coenzymes have similar affinities for the latter enzyme (Boers et al., 1971) so the chemical shift equivalence is expected. The reduced coenzyme, however, binds about 10^3 times more strongly to lactate dehydrogenase than does the oxidized coenzyme. It would thus appear that the pyrophosphate environment does not reflect this change in binding strength, and that one must look elsewhere on the coenzyme for the changed interactions which cause NADH binding to be so much stronger.

[1] The data indicate that there are at least two forms of the aggregated enzyme–coenzyme complex. One of these forms is in slow exchange with the unaggregated complex, and therefore a discrete peak at -1.9 ppm is observed. There is also a small amount of an aggregated species in rapid exchange with the unaggregated form, which leads to shifts intermediate between the shift of the higher molecular weight complex and that of the normal complex.

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Coenzyme Binding by Native and Chemically Modified Pig Heart Triphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase†

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ABSTRACT: The binding of TPNH to native and chemically modified pig heart TPN-dependent isocitrate dehydrogenase was studied by the techniques of ultrafiltration and fluorescence enhancement. A single site (per peptide chain) was found for TPNH with a dissociation constant (K_D = 1.45 μM) that is quantitatively comparable to the Michaelis constant. The oxidized coenzyme, TPN^+, weakens the binding of TPNH. The substrate manganous isocitrate also inhibits the binding of TPNH and, reciprocally, TPNH inhibits the binding of manganous isocitrate, suggesting that binding to the reduced coenzyme and substrate sites is mutually exclusive. Ultrafiltration experiments with carbonyl [^{14}C]TPN^+ revealed the existence of two sites with a dissociation constant (49 μM) more than ten times higher than the Michaelis constant. This observation excludes a random mechanism for isocitrate dehydrogenase or a sequential mechanism in which TPN^+ binds first. Four chemically modified isocitrate dehydrogenases have been prepared: enzyme inactivated by reaction of a single methionyl residue with iodoacetate, by modification of a glutamyl residue by glycinamide (in the presence of a water soluble carbodiimide), by reaction of four cysteines successively with 5,5′-dithiobis(2-nitrobenzoic acid) and potassium cyanide, or by addition of two cysteine residues to *N*-ethylmaleimide. These enzymes were tested for their ability to bind TPN^+, TPNH, and manganous isocitrate. In the cases of the cysteinyl and glutamyl-modified enzymes, inactivation appears to be due primarily to loss of the ability to bind the substrate manganous isocitrate. In contrast, the methionyl residue may participate in the coenzyme binding site or, more likely, may be involved in a step in catalysis subsequent to binding.

The active site of pig heart TPN-dependent isocitrate dehydrogenase (*threo*-Ds-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) has been explored by means of manganese and isocitrate binding experiments (Villafranca and Colman, 1972; Colman, 1969a). These studies have shown that manganese (Villafranca and Colman, 1972) and isocitrate (Colman 1969a) bind independently but weakly to isocitrate dehydrogenase but the binding of either is substantially enhanced when both are present. Analysis of kinetic data has indicated that the preferred substrate is the manganese–tribasic isocitrate complex (Colman, 1972a). In contrast to the extensive data on the interaction of the enzyme with isocitrate and manganese, little is known of the coenzyme binding properties of isocitrate dehydrogenase. A study of the interaction of TPNH with a partially purified enzyme preparation suggested the existence of two TPNH sites with extremely tight binding (dissociation constant approximately 10^{-8} M) (Langan, 1960). Qualitative experiments indicated competition with TPNH by TPN^+ and isocitrate in the presence of manganese (Langan, 1960). In addition to its role as coenzyme in the carboxylation of α-ketoglutarate, TPNH has been shown to be necessary for the detritiation of α-ketoglutarate in the presence of isocitrate dehydrogenase (Rose, 1960). The present investigation uses a homogeneous preparation of isocitrate dehydrogenase to examine quantitatively the binding of TPNH and the interaction between TPNH, TPN^+, and manganese–isocitrate. In addition, carbonyl-labeled [^{14}C]TPN^+ has been used to measure directly the affinity of the native enzyme for TPN. Binding data for oxidized and reduced coenzymes are compared with kinetic data in order to relate them with the possible reaction mechanisms.

Information about the function of amino acid residues at the active site can be obtained by examining the characteristics of enzymes which have been chemically modified at

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