turally modified flavins (modification being restricted to the isoalloxazine moiety of FAD) which indeed possess a higher AFR than the native enzyme (S. T. Olson and V. Massey, unpublished observations). Thus, enzyme reconstituted with 8-α-hydroxy-FAD had an AFR value of 166 and enzyme reconstituted with iso-FAD had an AFR of 126. While the modified natural enzymes do not contain either of these flavins, the possibility is clear that the variable AFR values of different enzyme preparations may reflect the content of flavins of unknown structure.

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Correlations between Tertiary Structure and Energetics of Coenzyme Binding in Pig Heart Muscle Lactate Dehydrogenase†

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ABSTRACT: Fluorescence, equilibrium dialysis, and microcalorimetric measurements have been performed on complex formation between pig heart muscle lactate dehydrogenase (EC 1.1.1.27) and a series of systematically modified nicotinamide adenine dinucleotide analogues to provide quantitative data for a discussion on energy–structure–function correlations. As a result of these studies, one can draw the conclusion that estimates of the relative stability of enzyme–ligand complexes on the mere basis of structural information on the macromolecule and its complexes with the ligand are likely to neglect contributions to the energy and entropy parameters, which stem from such processes as changes in solvation and conformation of both the free ligand and the macromolecule in the reaction. Since the reaction parameters reflect the differences between these states, information on hydrogen bonding and hydrophobic interaction schemes of the liganded and unliganded macromolecule alone is principally insufficient.

Detailed insight into the steric requirements of possible interactions between coenzyme and enzyme in the active center of dehydrogenases has been derived from comparative studies on various lactate dehydrogenase isozymes (Eventoff et al., 1977), on glyceraldehyde-3-phosphate dehydrogenases (Harris & Waters, 1976; Harris & Walker, 1977), and on alcohol dehydrogenase (Brändén, 1977; Brändén et al., 1975). Visualization of the tertiary structure does not, however, permit quantitative assessment of the energetic contributions of the various groups of the ligands involved in bond formation. Various approaches are possible in providing information on energetic details governing dehydrogenase–coenzyme complex formation. Two important approaches are (1) the utilization of a series of coenzyme fragments, the thermodynamic parameters of which are determined and compared to those of the natural coenzyme, and (2) the employment of the coenzyme itself which is modified chemically in a systematic fashion and analysis of the influence of the modifications on the overall energetic quantities. Results of studies on the energetics of binding of coenzyme fragments have been re-

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ported by Hinz & Schmid (1977), Schmid et al. (1978), Hinz et al. (1978), and Hinz & Schmidt (1979). Investigations employing a wide variety of coenzyme analogues with the objective of sorting out the molecular requirements for activity in the catalytic reaction have been described by Kaplan (1963a,b), Pfleiderer et al. (1964), Woenckhaus & Volz (1966), and Woenckhaus & Jeck (1969) and have been recently surveyed by Woenckhaus (1974). These studies provided a basis upon which to delineate the essentials for coenzyme activity. They did not cover, however, another important aspect of enzyme–analogue interaction, i.e., the correlation between the kinetic, structural, and energetic parameters of the reaction. Such a comparison requires knowledge of Gibbs free energies, enthalpies, entropies, and heat capacity changes. In this communication we report these data as the result of thermodynamic analyses of the reactions with pig heart muscle lactate dehydrogenase and a number of systematically modified coenzyme analogues. Binding constants have been obtained by equilibrium dialysis and fluorometric titrations, and the reaction enthalpies have been determined by direct calorimetric measurements in a flow calorimeter (Weber & Hinz, 1976).

Experimental Section

Materials

Pig heart muscle LDH[1] (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), NAD^+ (Grade I), $TNAD^+$, $APAD^+$, NADH, and AMP were purchased from Boehringer, Mannheim, Germany. Reagent-grade potassium hydrogen phosphate and potassium dihydrogen phosphate were obtained from E. Merck, Darmstadt, Germany. All other chemicals were reagent grade. Quartz-distilled, demineralized water was used for solution preparations.

LDH was supplied as a 1% crystalline suspension in ammonium sulfate. The enzyme crystals were collected from the suspension following centrifugation at 18 000 rpm for 15 min at 2 °C. The pellet was dissolved in 0.2 M phosphate buffer, pH 7.0. Remaining traces of ammonium sulfate were removed from the enzyme solution by passage through a Sephadex G-25 column. The enzyme was eluted with previously degassed 0.2 M phosphate buffer, pH 7.0. The enzyme concentration was based on a molecular weight of 140 000 and an absorption coefficient $E = 1.4$ cm^2 mg^{-1} at 280 nm. The ratio of the absorption at 280 nm to 260 nm was 1.88 ± 0.2.

The preparations of the analogues and fragments of NAD^+ were as follows: 2-(3-acetylpyridino)ethyl adenosine pyrophosphate, 5-(3-acetylpyridino)pentyl adenosine pyrophosphate, and 6-(3-acetylpyridino)hexyl adenosine pyrophosphate (Jeck, 1975); 3-(3-acetylpyridino)propyl adenosine pyrophosphate (Woenckhaus et al., 1970, 1973); 4-(3-acetylpyridino)butyl adenosine pyrophosphate (Jeck et al., 1973); NMD^+ (Woenckhaus, 1964); NMN^+ and $NMNPR^+$ (Woenckhaus & Scherr, 1973); NMD^+ (Pfleiderer et al., 1964); NBD^+ (Woenckhaus, 1964). $APAD^+$ was further purified by passing it through a long Dowex 1 formate column. $TNAD^+$ was used directly as obtained.

Concentrations of the coenzyme, analogues, and fragments were determined spectrophotometrically with a Cary 118 UV spectrophotometer by using the following absorption coefficients: NAD^+, 18×10^3 M^{-1} cm^{-1} at 260 nm; NADH, 6.23×10^3 M^{-1} cm^{-1} at 340 nm (Beisenherz et al., 1955); those of the ethyl, propyl, butyl, pentyl, and hexyl derivatives of (3-acetylpyridino)-*n*-alkyl adenosine pyrophosphate were 17.5, 18.1, 17.2, 18.4, and 17.9×10^3 M^{-1} cm^{-1}, respectively, at 259 nm (except for ethyl at 260 nm) and pH 9.5 (Jeck, 1975); $APAD^+$ 16.4×10^3 M^{-1} cm^{-1} at 260 nm and pH 9.5 (Jeck, 1975); $NMNPR^+$, $6.80 \pm 0.05 \times 10^3$ M^{-1} cm^{-1} at 265 nm and pH 9.5 (Jeck, 1975); NMN^+, 4.6×10^3 M^{-1} cm^{-1} at 266 nm and pH 7.0 (Jeck et al., 1974); NMD^+, 23.2×10^3 M^{-1} cm^{-1} at 310 nm and pH 9.5 (Pfleiderer et al., 1964). A 0.5 M glycine (NaOH) buffer was used at pH 9.5. NBD^+ concentrations were based on weight.

pH values of the buffer solutions were determined by means of a WTW pH meter (Digi 510, Wiss.-Tech. Werkstätten, Weilheim, Germany) and adjusted at the temperature of the measurements.

Methods

Equilibrium dialysis measurements were performed in a temperature-controlled walk-in room set at 25 °C. Self-constructed cells with individual compartment volumes of 0.25 cm^3 were separated by ordinary dialysis tubing (Myer & Schellman, 1962). A small air bubble remained after the addition of 0.2 mL of solution to each compartment, which aided in mixing the solutions during the mechanical rotation of the cells. Equilibrium was reached within 7 h. Experiments in which the enzyme and analogue were placed in opposite compartments and in which the enzyme and analogue were placed in the same compartment gave the same binding constant and therefore demonstrated the achievement of equilibrium. The concentration of the free analogue was determined by UV absorption.

Fluorescence Titrations. The binding constants for 4-(3-acetylpyridino)butyl adenosine pyrophosphate and 6-(3-acetylpyridino)hexyl adenosine pyrophosphate were determined through fluorescence titrations. Fluorescence was measured with a Hitachi Perkin-Elmer MPF 2A fluorometer. The excitation and emission wavelengths were 328 and 435 nm, respectively. The temperature of the cell compartment was controlled by a Haake KT 33 thermostat and measured in the cells with a YSI Model 46 TUC Tele-thermometer. Titrations of 2 mL of a 1.5 μM LDH solution in the absence and in the presence of varying concentrations of analogue with 1 mM NADH were performed at 25 °C in 0.2 M phosphate, pH 7.0. Apparent binding constant values for NADH were derived from the titration curves through a linearization procedure (Stinson & Holbrook, 1973). The equilibrium dissociation constant K_{analogue} for the binding of the analogue to LDH can be calculated from the slope of the equation

$$K'_{\text{NADH}} = K_{\text{NADH}} + \frac{K_{\text{NADH}}}{K_{\text{analogue}}}[\text{analogue}] \qquad (1)$$

by plotting K'_{NADH}, the apparent dissociation constant, vs. the total analogue concentration. The intrinsic equilibrium dissociation constant, K_{NADH}, was 3.9 μM at 7.0 and 25 °C (Schmid et al., 1976). The analogue dissociation equilibrium constants listed in Table I, being fairly large, require the utilization of high analogue concentrations. This requirement is made evident by inspection of the second right-hand term of eq 1 which is responsible for making K'_{NADH} greater than K_{NADH}. In addition to the use of high analogue concentrations, it would be advantageous to use a compound with fluorescence properties similar to NADH but which possesses a weaker binding constant. The reduced form of 4-(3-acetyl-

[1] Abbreviations used: LDH, lactate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; $APAD^+$, 3-acetylpyridine adenine dinucleotide; NMD^+, nicotinamide benzimidazole dinucleotide; $TNAD^+$, thionicotinamide adenine dinucleotide; NMN^+, nicotinamide mononucleotide; $NMNPR^+$, nicotinamide diribose 5′,5″-pyrophosphate; AMP, adenosine 5′-monophosphate.

Table I: Thermodynamic Quantities for the Binding of NAD^+ Analogues to LDH in 0.2 M Phosphate Buffer, pH 7, at 25 °C

analogue	analogue[a] (mM)	LDH (μM)	ΔQ[b] (kcal/mol of enzyme)	K_D[c] (μM)	ΔH (kcal/mol of enzyme)	ΔG (kcal/mol of enzyme)	ΔS [cal/[(mol of enzyme) deg]]
nicotinamide adenine dinucleotide, NAD^+	3.06	18.7	−20.8 ± 0.9	325[f]	−23.1 ± 0.9 −23.2 ± 0.8[f] −24.5 ± 0.8[f] −24.4 ± 0.8	−19.0	−13.5
thionicotinamide adenine dinucleotide, $TNAD^+$	3.66	17.9	−20.6 ± 1.7	350	−22.6 ± 1.7	−18.9	−12.6
3-acetylpyridine adenine dinucleotide, $APAD^+$	1.37	17.2	−29.2 ± 1.6	100 135 170[d]	−32.2 ± 1.6	−21.1	−37.2
2-(3-acetylpyridino)ethyl adenosine pyrophosphate	4.88	21.2	−4.6 ± 1.1	2750	−7.2 ± 1.6	−14.0	22.6
3-(3-acetylpyridino)propyl adenosine pyrophosphate	5.25	21.0	−8.0 ± 1.5	1650	−10.6 ± 1.5	−15.2	15.6
4-(3-acetylpyridino)butyl adenosine pyrophosphate	6.70	23.2	−13.5 ± 1.3	700 1100[e]	−14.9 ± 1.3	−17.2	7.7
5-(3-acetylpyridino)pentyl adenosine pyrophosphate	4.82	21.6	−15.1 ± 1.9	500	−16.7 ± 1.9	−18.0	4.4
6-(3-acetylpyridino)hexyl adenosine pyrophosphate	5.70	21.2	−18.7 ± 2.1	300 160[e]	−19.7 ± 2.1	−19.2	−1.6
nicotinamide 6-mercaptopurine dinucleotide, NMD^+	3.89	12.0	−9.8 ± 2.0	300	−10.6 ± 2.0	−19.2	29.0
nicotinamide benzimidazole dinucleotide, NBD^+	4.89	19.3	−9.6 ± 1.1	600	−10.8 ± 1.1	−17.6	22.8

[a] Analogue and enzyme concentrations were the final concentrations after mixing in the calorimeter. [b] The observed experimental heat of reaction; error limits are expressed as standard deviation. [c] K_D, the intrinsic dissociation equilibrium constant, was determined by competitive binding with the reduced form of 4-(3-acetylpyridino)butyl adenosine pyrophosphate by fluorescence detection. [d] Determined by equilibrium dialysis. [e] Determined by competitive binding with NADH by fluorescence detection. [f] Values obtained by Schmid et al. (1976).

pyridino)butyl adenosine pyrophosphate has a K_D value of 14 μM at pH 7.0 and 25 °C which is ~4 times weaker binding than NADH. This compound was used to determine the majority of the dissociation equilibrium constants listed in Table I.

Calorimetry. Calorimetric measurements were carried out at 10, 25, and 35 °C on a modified Beckman Model 190 flow microcalorimeter (Weber & Hinz, 1976). Pulsed-flow experiments were generally performed with pulses of 50-s duration. Under these conditions ~0.3 mL of the enzyme and analogue solutions was delivered into the reaction cell per pulse. The calibration constant of the flow calorimeter was determined by sucrose dilution at 25 °C (Gucker et al., 1939) and by neutralization of HCl at 10, 25, and 35 °C (Grenthe et al., 1970).

In most cases the heat at saturation, ΔH, where 4 mol of anlogue is bound to 1 mol of enzyme, was not directly measured, but rather the heat for a limited extent of reaction, ΔQ, was measured. This situation stemmed from the fact that some of the analogues bind rather weakly to the enzyme. The experimental approach utilized was to use the highest possible analogue concentration so as to maximize the degree of binding to the enzyme. However, limitations were imposed because of the large heats of dilution observed at these high analogue concentrations. Therefore, a compromise in analogue concentration was made in order to decrease the contribution of the analogue heat of dilution to the overall observed heat of reaction. The binding constants given in Table I were used in evaluating the enthalpies from the observed heats at the specified enzyme and analogue concentrations. Equilibrium constants at 10 °C shown in Table II were computed through the integrated form of the van't Hoff equation

$$\ln \frac{K_{T_2}}{K_{T_1}} = \frac{1}{R}\left(\frac{\Delta H_{T_2}}{T_2} - \frac{\Delta H_{T_1}}{T_1}\right) - \frac{\Delta C_p}{R} \ln \frac{T_2}{T_1} \qquad (2)$$

where K, ΔH, and ΔC_p were the dissociation equilibrium constant, the association enthalpy, and the association heat capacity which was assumed to be independent of temperature, respectively.

Results

The energetics of NAD^+ analogues, as well as a couple of fragments, binding to LDH were investigated by direct calorimetric measurements and supplemented in most cases by fluorometric methods for determining the equilibrium binding constants. The adenosine monophosphate and the nicotinamide mononucleotide domains constitute the NAD^+ coenzyme molecule. Modifications of the adenine site through the synthesis of NMD^+ and NBD^+ and the preparation of the fragment, $NMNPR^+$, without the adenine ring were studied (Figure 1). NMD^+, NBD^+, and $NMNPR^+$ were individual examples of the following classes: analogue containing a different purine system, analogue with the substitution of the nonfunctional (with respect to direct involvement in the oxidation–reduction processes) ring system, and a coenzyme fragment.

Modifications of the nicotinamide site were realized in the analogues $TNAD^+$ and $APAD^+$ (Figure 2). In addition, derivatives of $APAD^+$ in which the ribose ring adjacent to the pyridine ring was substituted by straight aliphatic hydrocarbon chains of various lengths were studied (Figure 3). The series of ω-(3-acetylpyridino)-*n*-alkyl adenosine pyrophosphate compounds investigated where n = 2, 3, 4, 5, and 6 which were the ethyl, propyl, butyl, pentyl, and hexyl derivatives, respectively.

Analogues Containing Different Purine Systems. NMD^+ was a coenzyme analogue with the substitution of the adenine by another purine derivative, namely, the replacement of the 6-amino group by a 6-mercapto group (Figure 1). The catalytic properties as exhibited by V_{max} = 14000 and K_M = 300 μM were similar to those of the coenzyme NAD^+, where V_{max} = 15000 and K_M = 100 μM. Here and henceforth, all stated V_{max} values, expressed in moles of NADH per mole of LDH

Table II: Thermodynamic Quantities for the Binding of NAD^+ Analogues to LDH in 0.2 M Phosphate Buffer, pH 7, at 10 °C

analogue	analogue[a] (mM)	LDH (μM)	ΔQ[b] (kcal/mol of enzyme)	K_D[c] (μM)	ΔH (kcal/mol of enzyme)	ΔG (kcal/mol of enzyme)	ΔS [cal/[(mol of enzyme) deg]]
nicotinamide adenine dinucleotide, NAD^+					-19.3 ± 0.8[d]		
thionicotinamide adenine dinucleotide, $TNAD^+$	4.04	20.6	−21.2	216	−22.4	−19.0	−11.9
3-acetylpyridine adenine dinucleotide, $APAD^+$	1.72	17.6	-27.7 ± 1.1	68	-28.8 ± 1.1	−21.6	−25.6
2-(3-acetylpyridino)ethyl adenosine pyrophosphate	5.00	22.4	-6.0 ± 1.9	2300	-8.8 ± 1.9	−13.7	17.2
3-(3-acetylpyridino)propyl adenosine pyrophosphate	4.42	23.2	-6.8 ± 1.9	1371	-8.9 ± 1.9	−14.8	20.8
4-(3-acetylpyridino)butyl adenosine pyrophosphate	4.73	22.4	-12.3 ± 1.8	506	-13.6 ± 1.8	−17.1	12.2
5-(3-acetylpyridino)pentyl adenosine pyrophosphate	2.22	26.5	-14.1 ± 1.3	332	-16.3 ± 1.3	−18.0	6.1
6-(3-acetylpyridino)hexyl adenosine pyrophosphate	5.52	20.7	-17.1 ± 1.8	199	-17.7 ± 1.8	−19.2	5.1
nicotinamide 6-mercaptopurine dinucleotide, NMD^+	2.52	23.3	-6.1 ± 1.0	237	-6.7 ± 1.0	−18.8	42.7
nicotinamide benzimidazole dinucleotide, NBD^+	3.96	22.0	-7.9 ± 0.8	471	−8.9	−17.2	29.6

[a] Analogue and enzyme concentrations were the final concentrations after mixing in the calorimeter. [b] The observed experimental heat of reaction; error limits are expressed as standard deviation. [c] K_D, the dissociation constant for the binding of one ligand, was calculated with the K_D and ΔH values at 25 °C and an estimated value of ΔH at 10 °C via the van't Hoff relationship. [d] Values taken from Hinz & Schmid (1977).

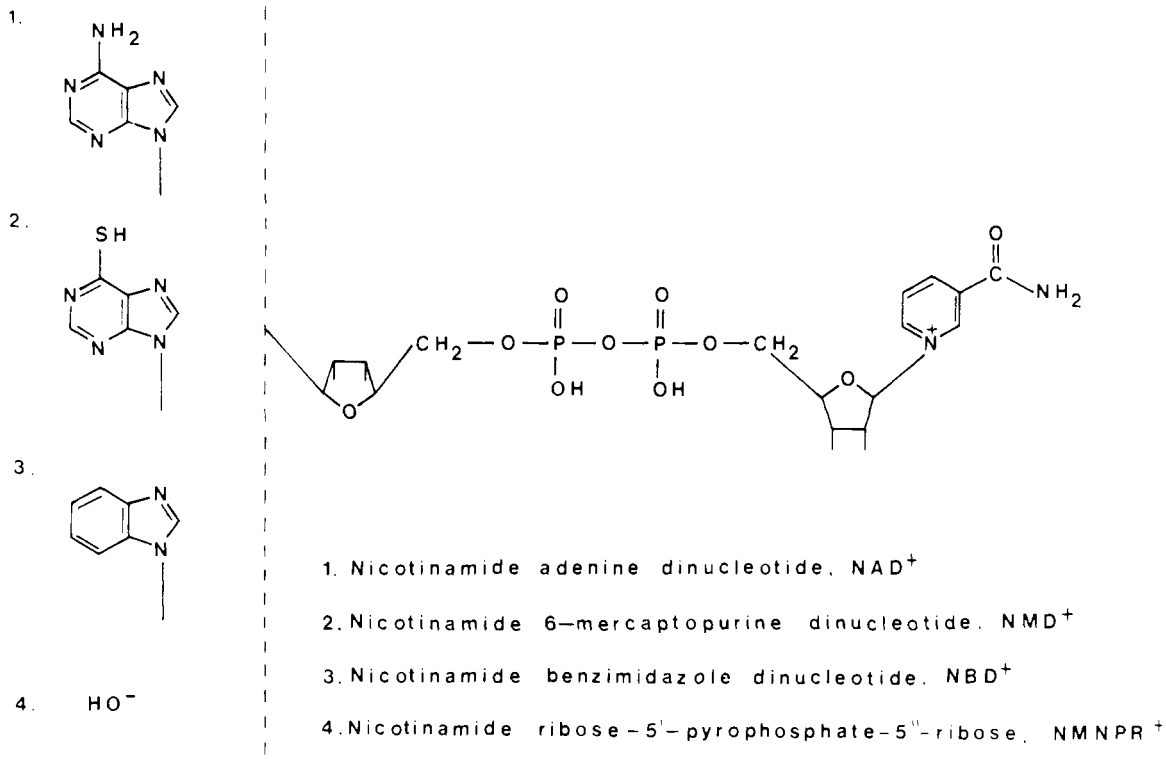

FIGURE 1: Nicotinamide adenine dinucleotide, NAD^+, analogues with modifications of the adenine site.

per minute, and K_M values were determined at 25 °C and pH 9.5. Despite this comparison in catalytic properties, the enthalpy of NMD^+ binding was a factor of 2 smaller than that of the natural coenzyme NAD^+ (Table I). The amino group in position 6 apparently has no particular specific interaction with the enzyme and points outward into the solution (Holbrook et al., 1975). The enthalpic difference between NMD^+ binding and NAD^+ binding may reflect the difference in electron density of the purine ring system.

Analogues with Substitution of the Nonfunctional Ring System. The purine ring is made up of a pyrimidine and an imidazole ring fused together. A benzene ring fused to an imidazole ring constitutes benzimidazole which was found in the analogue NBD^+ (Figure 1). In comparison to NAD^+, the 6-amino group was absent and a homocyclic benzene ring was substituted for the heterocyclic pyrimidine ring. The value of V_{max} was 15 000, and K_M was 600 μM. The aromatic character was maintained; however, the interaction between Asp-53 and N-3 via a hydrogen bond and the interaction between Tyr-85 and N-1 via a hydrogen bond found with the coenzyme NAD^+ by X-ray crystallographic investigations (Holbrook et al., 1975) were lacking in NBD^+. NBD^+, like NMD^+, interestingly possessed approximately half the enthalpy of binding found for NAD^+ (Table I).

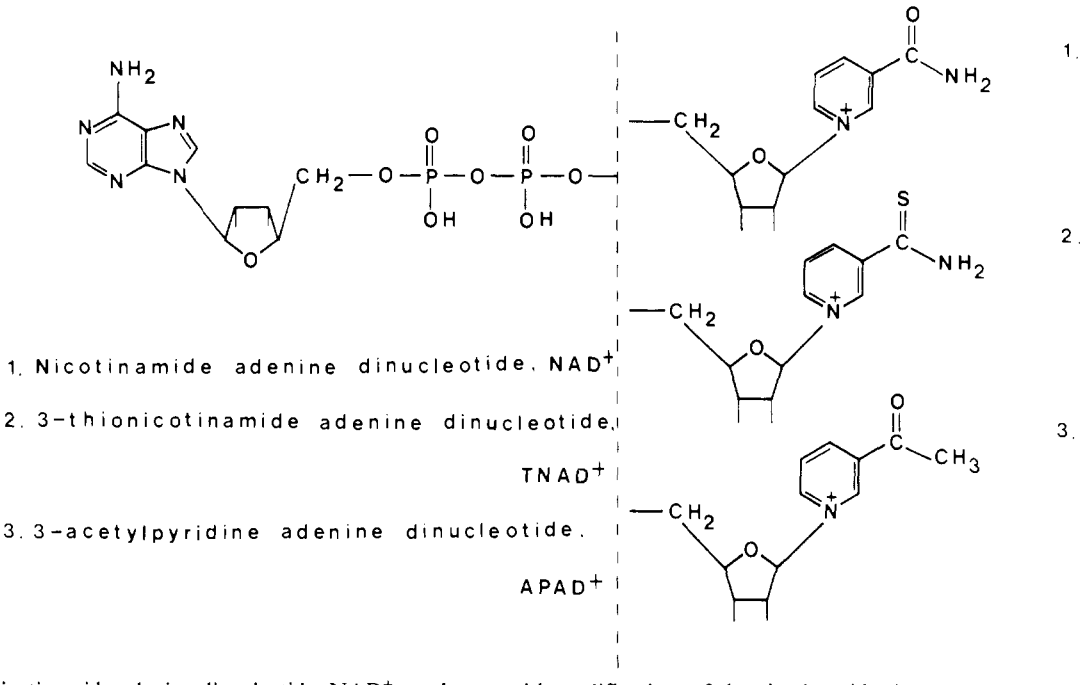

FIGURE 2: Nicotinamide adenine dinucleotide, NAD^+, analogues with modifications of the nicotinamide site.

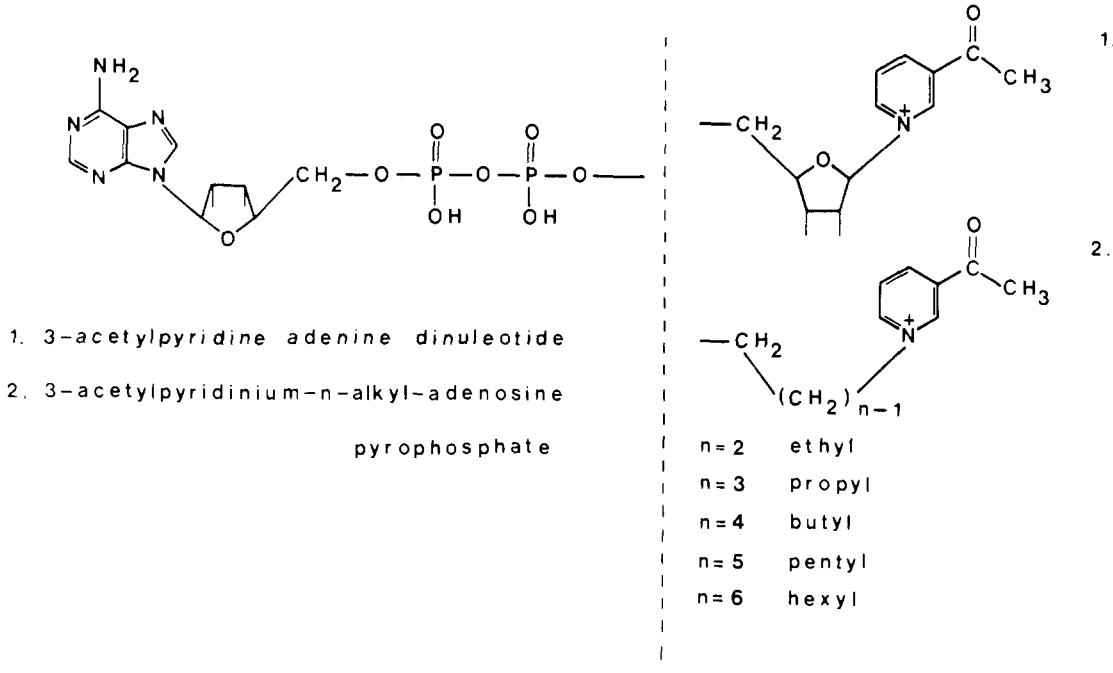

FIGURE 3: 3-Acetylpyridine adenine dinucleotide, $APAD^+$, analogues in which the ribose on the nicotinamide site is substituted by methylene chains of varying lengths.

Analogues with Substitution on the Pyridine Ring. A single-atom replacement of the carbonyl-group oxygen by a sulfur atom in the acid amide group attached at position 3 on the pyridine ring gave the analogue $TNAD^+$ (Figure 2). The energetic picture of $TNAD^+$ binding was the same as that of the natural coenzyme (Table I). However, the relative rate of reduction of $TNAD^+$ compared to NAD^+ was 0.41 ± 0.1. The redox potential, E_0', at pH 7 and 20 °C was −285 mV in contrast to −316 mV for NAD^+ (Kaplan & Anderson, 1959). Replacement of the amino group in the acid amide attached at position 3 on the pyridine ring by a methyl group gives rise to 3-methyl ketone side group, that is, the 3-acetyl group (Figure 2). This analogue, $APAD^+$, possessed a noticeably different redox potential E_0', at pH 7 and 20 °C, of −257 mV in comparison to −316 mV for NAD^+. The amide group in NAD^+ apparently provided the pyridine ring with the appropriate redox potential through the conjugated double bond. $APAD^+$ showed little coenzyme activity, as reflected by a V_{max} = 140 and a K_M = 25 μM. Despite the poor catalytic activity, the interaction of $APAD^+$ with LDH was more exergonic, and the enthalpy of binding was more favorable than that of NAD^+ (Table I).

Analogues Where the Pyridine Ribose of $APAD^+$ Was Replaced by Aliphatic Chains. The parent compound in this study was $APAD^+$. The replacement of the ribose adjacent to the pyridine ring with aliphatic straight chains of varying lengths provided the following derivatives: ethyl, propyl, butyl, pentyl, and hexyl, where n = 2, 3, 4, 5, and 6, respectively (Figures 3 and 4). The distances between the oxygen and nitrogen in Figure 4 are as follows: n = 2, 3.45 Å; n = 3, 4.87 Å; n = 4, 6.07 Å; n = 5, 7.37 Å; n = 6, 8.59 Å (Jeck, 1975). These distances refer to the extended, staggered aliphatic chain

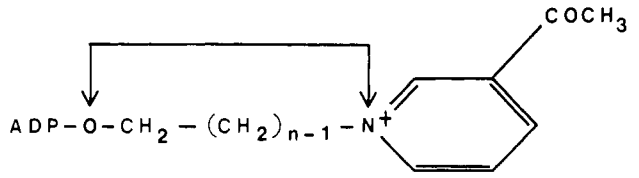

FIGURE 4: Schematic representation of 3-acetylpyridine adenine dinucleotide, APAD$^+$.

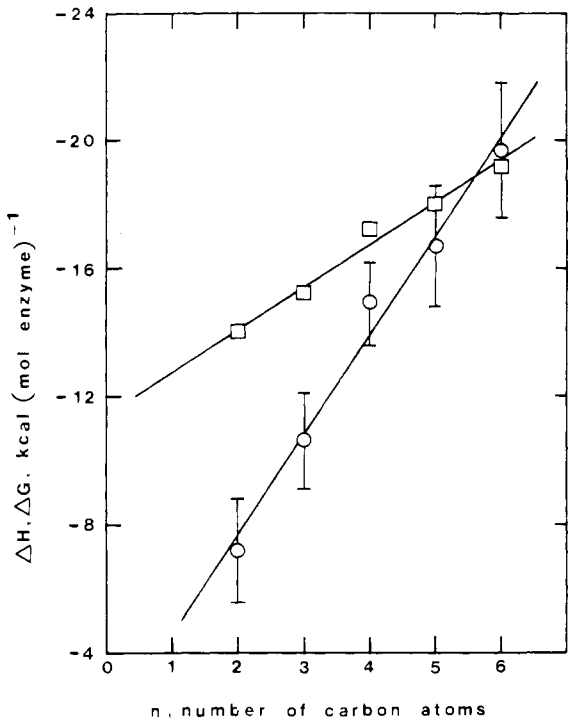

FIGURE 5: Binding enthalpies (○) and standard free energies (□) of (3-acetylpyridino)-*n*-alkyl adenosine pyrophosphate compounds with LDH in 0.2 M phosphate, pH 7.0, at 25 °C where n = 2, 3, 4, 5, and 6 are ethyl, propyl, butyl, pentyl, and hexyl derivatives, respectively. The experimental conditions are given in Table I. Enthalpy error limits are expressed as standard deviation.

and therefore reflect a maximum possible distance. For NAD$^+$ the distance between the O and the N was estimated to be 6.02 Å, which compared with that of the 4-(3-acetylpyridino)butyl adenosine pyrophosphate. Despite the distance between the ADP portion and the pyridine ring for NAD$^+$ and the butyl analogue being the same, the thermodynamic quantities (Table I and Figure 5) did not particularly suggest that the butyl derivative possessed the optimum distance. One possible explanation of this discrepancy is the assumption that the actual conformation of the aliphatic chain on the enzyme is not the maximum extended staggered conformation but some intermediate conformation. In such a case no expectation of an optimum fit of the coenzyme to the binding domain can be based on the distance calculations. However, a linear relationship appeared to exist both between the enthalpy of binding and between the free energy of binding with the number of methylene groups, respectively. (Figures 5 and 6). The slopes of the enthalpy curves (Figures 5 and 6) were −3.1 (25 °C) and −2.5 (10 °C), and that of the standard free energy curve (Figure 5) was −1.3 kcal [(mol of enzyme) methylene group)]$^{-1}$ at 25 °C. The derivatives differed from APAD$^+$ in that the redox potentials, E_0', were approximately the same as that of NAD$^+$ (Jeck, 1975). However, poor coenzyme activities were exhibited by propyl, butyl, and pentyl derivatives which had K_M values in the mM range and V_{max} values of 0, 5, and 0, respectively (Jeck, 1975).

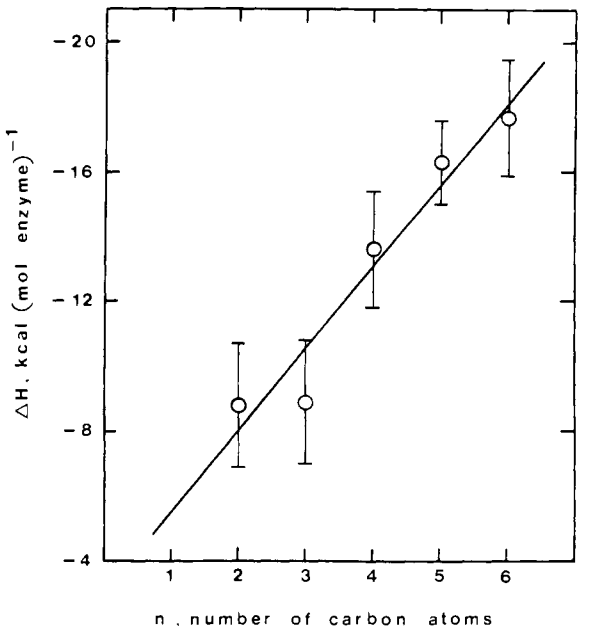

FIGURE 6: Binding enthalpies of (3-acetylpyridino)-*n*-alkyl adenosine pyrophosphate compounds with LDH in 0.2 M phosphate, pH 7.0, at 10 °C where n = 2, 3, 4, 5, and 6 are the ethyl, propyl, butyl, pentyl, and hexyl derivatives, respectively. The experimental conditions are given in Table II. Enthalpy error limits are expressed as standard deviation.

NMNPR$^+$ and NMN$^+$ Fragments. NMNPR$^+$ which was devoid of the adenine group had a V_{max} value of 500 and K_M = 6000 μM (Jeck, 1975). The observed heat of reaction, ΔQ, between 8.3 mM NMNPR$^+$ and 37.1 μM LDH was 0.0 kcal/mol of enzyme. Either the NMNPR$^+$ binding was extremely weak or the enthalpy of binding was very small or both conditions may have applied. Apparently the presence of the adenine moiety plays an important function in terms of coenzyme binding. NMN$^+$, which was NAD$^+$ without the AMP constituent, gave a heat of reaction, ΔQ, value of +5.6 ± 1.7 kcal/mol of enzyme where LDH = 17 μM and NMN = 70 mM. Due to the unusually high NMN$^+$ concentration, the observed heat may have been due to secondary binding. Another experiment in which both NMN (72.5 mM) and LDH (23.3 μM) were allowed to react in the presence of saturating amounts of tight-binding NAD (402 μM) gave a ΔQ value of +4.5 ± 1.1 kcal/mol of enzyme. Apparently the observed heat was due to some other phenomena other than that of NMN$^+$ binding to the coenzyme binding site.

It has been reported that NMN$^+$ alone does not bind to LDH, but it can bind with the enzyme in the presence of AMP (McPherson, 1970). The binding equilibrium constant, K_D, for AMP to LDH is 1.2 mM, and the enthalpy of binding is −12.8 kcal/mol of enzyme (Hinz & Schmid, 1977). This enthalpy value was approximately half that for NAD binding. An experiment was performed in which approximately equimolar quantities of NMN$^+$ and AMP were allowed to react with LDH. The concentrations were 11.4 mM, 11.2 mM, and 16.7 μM for NMN$^+$, AMP, and LDH, respectively. The observed heat, ΔQ, was −12.5 ± 0.4 kcal/mol of enzyme. From this preliminary experiment it appeared that the above heat was due solely to AMP binding.

Temperature Dependence of the Enthalpy of Binding. The temperature dependence for the enthalpy of association of NAD$^+$ with LDH was −334 cal/[deg (mol of enzyme)] (Schmid et al., 1976). In contrast, the ω-(3-acetylpyridino)-*n*-alkyl adenosine pyrophosphate compounds gave ΔC_p values at 17.5 °C of −45, −66, −87, and −110 cal/[(mol

of enzyme) deg] for the propyl, butyl, pentyl, and hexyl derivatives, respectively. The ΔC_p values were calculated using the least-square enthalpy of binding values taken from Figures 5 and 6. The ΔC_p values for $APAD^+$, NMD^+, NBD^+, and $TNAD^+$ were −227, −260, −127, and −13 cal/[deg (mol of enzyme)], respectively. None of the coenzyme analogues possessed a ΔC_p value as large as that of the natural coenzyme, NAD^+.

Discussion

All studies on the interaction of coenzyme analogues or fragments and lactate dehydrogenases have had the objective of providing information, which goes beyond a gross characterization of the binding in order to depict a molecular or even an atomic picture of the various interactions between the coenzyme and the enzyme (Everse & Kaplan, 1973). X-ray studies have contributed a lot to render the steric requirements and the mode of binding of the coenzyme more understandable (Holbrook et al., 1975; Eventoff et al., 1977), while kinetic investigations delineated the reaction sequence (Holbrook et al., 1975). However, the intriguing goal of assigning specific energy contributions to individual groups of the coenzyme still seems to be a long way off in view of the results obtained by calorimetric studies on the binding of coenzyme fragments to lactate and alcohol dehydrogenase. No simple additive energy increments characteristic of the interaction of each group with the enzyme could be delineated from these investigations nor can they realistically be expected. (Hinz & Schmid, 1977; Schmid et al., 1978; Hinz et al., 1978; Hinz & Schmidt, 1979). Rather, it has to be anticipated that any structural change introduced in the coenzyme perturbs a delicate energetic balance in a complex manner, since it involves not only an alteration of coenzyme properties such as electronegativity, redox potentials, and resonance structures of particular groups but also its solvation on and off the enzyme and its ability to trigger conformational changes in the macromolecule. These alterations in the interaction of the enzyme with the analogue relative to complex formation with the natural coenzyme are more pronouncedly reflected in the reaction enthalpies and heat capacity changes than in the Gibbs free energies. Since the energy parameters are not predictable by theoretical calculations nor deducible from structural considerations, they have to be determined by direct calorimetric investigations.

Analogues with Modifications of the Adenine Site. Exchange of the amino group of adenine by a thiol group leads to a surprisingly small binding enthalpy of approximately −11 kcal/mol of enzyme as compared to the −24 kcal/mol of enzyme obtained with NAD^+ (Table I). This result is particularly remarkable in view of the X-ray studies, which suggest no involvement of the amino group of adenine in any visible specific bond formation. Although this conclusion is only valid for the ternary complex, it is rather unlikely that the adenine moiety in the binary complex assumes a markedly different orientation on the enzyme from that in the ternary complex. Thus, the large decrease in the enthalpic contribution to stability is likely to result from perturbation of the electronic structure of the purine ring system due to the presence of the sulfur atom. This conclusion is supported by the results obtained with nicotinamide benzimidazole dinucleotide, NBD^+ (Table I), in which the pyrimidine is replaced by a benzene ring. The catalytic and energetic parameters of NMD^+ and NBD^+ at 25 °C were rather similar (NMD^+, V_M = 14 000, K_M = 300 μM, ΔH = −10.6 kcal/mol, and K_D = 300 μM; NBD^+, V_M = 15 000, K_M = 600 μM, ΔH = −10.8 kcal/mol, and K_D = 600 μM) and comparable to those of NAD^+ (V_M = 15 000, K_M = 100 μM, ΔH = −24 kcal/mol, and K_D = 325 μM) except for the strong difference in reaction enthalpy. Another divergence becomes apparent when the binding studies were performed at 10 °C. The change of heat capacity of NMD^+ was −260 cal/[(mole of enzyme) K]; that of NBD^+ was −127 cal/[mol of enzyme) K]. The ΔC_p value for the reaction with the unmodified coenzyme NAD^+ was −334 cal/[(mol of enzyme) K].

These values are very difficult to rationalize in terms of induced structural or hydration changes of the macromolecule or of the coenzyme itself, due to the following reasons. (1) Although it may be inferred that the analogues bind in a similar manner to the enzyme, their solution structure is completely unknown. Their equilibrium distributions between extended and stacked conformations may differ strongly, as well as the variation with temperature of those equilibrium constants. (2) There is no information on possible differences in the extent of hydration of the analogues nor do estimates exist of changes in the thermodynamic quantities involved in the transfer from aqueous solution to the hydrophobic vicinity experienced by the adenine moiety on the enzyme (Holbrook et al., 1975). Since the thermodynamic parameters refer to the difference between these states all such contributions may be reflected in the data.

Analogues with Modifications of the Nicotinamide Site. The single-atom replacement of the carbonyl-group oxygen by a sulfur atom in the acid amide group of the pyrimidine ring ($TNAD^+$) leads to a considerable decrease of the rate of reduction as well as to a less negative redox potential of the analogue compared to that of NAD^+. The thermodynamic parameters, however, are practically identical within experimental error. This is to be expected, since though it is likely that an exchange of an oxygen atom by a sulfur in the amide group attached at position 3 on the pyrimidine ring influences the electron distribution in the 4 position, thereby altering the redox potentials, it would be difficult to conceive that such a change results in significant alterations of the thermodynamic binding parameters. This conclusion is in agreement with the X-ray studies (Eventoff et al., 1977) which show no apparent involvement of the oxygen of the acid group in binding interactions. What is, however, puzzling is the lack of any noticeable heat capacity change on binding. In view of the very good agreement of Gibbs free energies, enthalpies, and reaction entropies, this result cannot be rationalized. Therefore, for these reactions the suggestion appears somewhat less convincing (Yang & Rupley, 1979) that some of the negative heat capacity changes detected when NAD^+ binds to dehydrogenases may be understood merely in terms of hydration changes of the enzyme and coenzyme surface. Although undoubtedly contributions to ΔC_p may often stem from this source, the considerable differences in heat capacity changes when NAD^+ binds to pig heart or pig skeletal muscle isozyme of lactate dehydrogenase [−84 and −122 cal/[(mol of binding site) K], respectively (Hinz et al., 1978)] are difficult to understand. The same reasoning applies to the ΔC_p changes involved in the binding of NADH [−167 and −325 cal/[(mol of binding site) K], respectively (Hinz et al., 1978)] or adenosine [0 and −259 cal/[(mol of binding site) K], respectively (Hinz & Schmidt, 1979)] to the pig heart or skeletal muscle lactate dehydrogenase unless one would assume a different mode of binding of the coenzyme for the two isozymes with significantly higher hydration of both the binding domain and the coenzyme in the heart muscle dehydrogenase–coenzyme complex.

The complex nature of the interactions between the coenzyme or analogue and the enzyme was strikingly demon-

strated by the thermodynamic parameters for complex formation between 3-acetylpyridine adenine dinucleotide, $APAD^+$, and pig-heart lactate dehydrogenase. The replacement of the amino group in the acid amide at position 3 on the pyridine ring by a methyl group not only results in a more positive redox potential but also causes an unexpected improvement in the binding constant and a 34% increase in the favorable binding enthalpy (−32.2 kcal/mol of enzyme against −24 kcal/mol of enzyme for NAD^+). The heat capacity change on binding is −227 cal/[(mol of enzyme) K], which is 30% smaller than that obtained when NAD^+ binds. The more negative ΔG and ΔH values with respect to NAD^+ binding furnish an amazing enigma if one follows the enumeration of binding interactions derived from the X-ray picture for the tertiary complex (Eventoff et al., 1977). According to the diagrammatic representation [Figure 2 of Eventoff et al. (1977)], the hydrogen atoms of the amine group of the acid amide have the possibility of participating in hydrogen-bond formations with Ser-163 or Ser-139. Either of these possible interactions should be absent in the $APAD^+$–enzyme complex which leads one to expect less favorable binding and a less negative enthalpy; however, just the opposite is found. This experimetanl finding demonstrates that, in general, binding interactions derived from X-ray studies on the mere basis of distances provide too simplistic a picture since the energy and free energy values are the differences between a variety of favorable and unfavorable energetic and entropic contributions involved in the binding process, the individual values of which can be large.

Analogues Where the Pyridine Ribose of 3-Acetylpyridine Adenine Dinucleotide, $APAD^+$, Was Replaced by Aliphatic Chains (Figure 4 and Table I). It was known from investigations of Jeck (1975) that these analogues exhibit a redox potential close to that of the coenzyme NAD^+, while $APAD^+$, the parent compound containing the ribose, showed a 19% more positive standard redox potential as determined according to Wallenfels & Diekmann (1959). All analogues except for the 4-(3-acetylpyridino)butyl adenosine diphosphate show, however, practically no coenzyme activity. Even the activity of this compound, in which the correct distances are realized, is only 0.03% of the activity which the enzyme assumes with NAD^+. There is no apparent correlation between the thermodynamic and catalytic properties. However, there is a striking regularity in the increase of affinity and favorable binding enthalpy with increasing number of CH_2 groups substituted for the nicotinamide ribose. If one assumes in a very approximative manner that the negative increment in binding Gibbs free energy per CH_2 group stems from hydrophobic dehydration associated with the transfer of the hydrated coenzyme analogue from solution onto the hydrophobic surface of the enzyme and if one further assumes that, other groups being equal, the difference in ΔG^0 originates solely from dehydration of the CH_2 spacers, one could calculate, according to Gill & Wadsö (1976), a Gibbs free energy gain of −3.7 kcal/mol of enzyme, i.e., −3.7 kcal/mol of four CH_2 groups, which compares favorably with the experimental observation of −3.1 kcal/mol of enzyme. Unfortunately, this interpretation appears to be invalidated by trying to rationalize the ΔH values under the same model assumptions. The negative ΔH increment of −0.325 kcal/mol per methylene group (the value results from division by 4 of the −1.3 kcal/mol of enzyme, since there are four binding sites) is difficult to visualize as stemming from dehydration, since solvation enthalpies of alkyl methylene groups have been measured to be negative [−0.86 kcal/mol of CH_2 (Konicek & Wadsö, 1971)]; dehydration enthalpies should therefore give positive contributions. Even if one regards the heats of solution more appropriate as quantities to use in these considerations, the sign for the expected ΔH values of dehydration is still opposite that of the experimental finding. Thus, one has to draw the conclusion that the agreement in sign and magnitude of the negative ΔG increments per methylene group is fortuitous.

Studies on $NMNPR^+$ and NMN^+ Fragments (Figure 1 and Tables I and II). The rationale for these studies was to determine thermodynamic parameters for the contribution of the adenine-containing side of the coenzyme, which has been commonly assumed to provide the major binding interactions (McPherson, 1970). A crucial experiment was to characterize the binding reaction between $NMNPR^+$, the coenzyme fragment which lacks the adenine group, and the enzyme. From Jeck's (1975) experiments it was known that this analogue exhibits 3% of the catalytic activity of NAD^+ at pH 9.5. Its Michaelis constant was K_M = 6 mM under those conditions. If the binding constant at pH 7 is comparable to the Michaelis constant at pH 9 and the reaction enthalpy is of similar magnitude to the values in Tables I and II, a heat effect should have been observed when mixing 8.3 mM $NMNPR^+$ and 37.1 μM enzyme solutions. However, the experimental finding was 0.0 kcal/mol of enzyme. Thus, besides weak binding only a very small heat effect can be associated with complex formation, which has to take place as judged on the basis of the weak catalytic activity of the analogue. This result is in complete agreement with a previous complementary study (Hinz & Schmid, 1977) in which the reaction between adenosine and pig heart muscle lactate dehydrogenase was analyzed. Complex formation between adenosine and the enzyme at 25 °C yielded an enthalpy identical with that of binding the unmodified coenzyme NAD^+. Thus, the full binding enthalpy of the coenzyme stems from association of the adenosine moiety with the enzyme.

At least in principle there is the possibility that other fragments devoid of the adenine part show a binding enthalpy and that the vanishingly small enthalpy of $NMNPR^+$ results from compensatory effects. Therefore, binding of nicotinamide mononucleotide, NMN^+, which lacks the adenine ribose and phosphate groups was studied. At very high concentrations of the reactants ([LDH] = 17 μM and [NMN^+] = 70 mM after mixing, an enthalpy value of +5.6 ± 1.7 kcal/mol of enzyme was obtained, the large error limits being due to the fact that the enthalpy is the small difference between two large quantities. Repetition of the experiment in the presence of saturating amounts of the coenzyme NAD^+ resulted in the same enthalpy value, which suggests that the observed heat effect does not originate from binding of NMN^+ to the coenzyme binding site. This result corroborates McPherson's (1970) finding that NMN^+ does not bind in the absence of AMP.

The inhibition studies of McPherson suggested that in the presence of AMP the enzyme has undergone a conformational change such as to generate a binding site for NMN^+. Although the ΔH value obtained in the calorimetric experiment of binding NMN^+ and AMP to pig heart lactate dehydrogenase was the same as that observed when AMP alone was reacted with the enzyme, the interpretation is not unequivocal. Even if NMN^+ binds to the enzyme–AMP complex, the end product is not the same as the NAD^+–enzyme complex, due to the difference in charges between the pyrophosphate configuration in NAD^+ and the two phosphate groups of NMN^+ and AMP, respectively. A similar situation has been discussed to explain the difference of AMP and ADP

binding to alcohol dehydrogenase (Hinz et al., 1978). Such a difference should also influence the solvation of NMN^+ and AMP compared to that of NAD^+, prior to complex formation, resulting in overall reaction energies for the fragments, which a priori cannot be assumed to be the same as those for NAD^+ binding.

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