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Interaction of Nicotinamide–Adenine Dinucleotide and Its Analogs with Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: Several structural analogs of NAD were tested for their effects on the properties of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. Of the 13 analogs tested only three were effective substrates: acetylpyridine-AD, thionicotinamide-AD, and deamino-NAD. Several other analogs including ADP-ribose, ADP, AMP, NMN, and pyridine carbaldehyde-AD were inhibitors competitive with NAD. The latter analog was also competitive with the substrate phosphoglyceraldehyde. Two of the analogs gave a spectrum with the enzyme similar to that observed with β-NAD. These compounds, acetylpyridine-AD and deamino-NAD, also showed anticooperative binding to the enzyme. Several analogs produced partial stabilization of the enzyme at 45°. In decreasing order of stabilization these are: β-NAD, ADP-ribose, thionicotinamide-AD, ADP, NMN. From these studies as well as determinations of the kinetic and binding constants of the analogs it is possible to deduce a probable conformation of the bound coenzyme and to specify the major points of contact between the coenzyme and the enzyme. A model of the enzyme–coenzyme complex is presented.

In recent years there has been interest in the unusual kinetic and binding properties of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (GPDH)[1] and the corresponding enzyme obtained from yeast. Kirschner *et al.* (1966) have reported that the yeast GPDH exhibits positive cooperative binding of the coenzyme NAD under some conditions while the muscle enzyme does not give cooperative binding. On the other hand, Conway and Koshland (1968) have shown that there is anticooperative binding of NAD to the muscle enzyme. In a more recent study Cook and Koshland (1970) have reexamined the binding of NAD to the yeast enzyme and have shown that there is positive cooperativity in the first half of the curve and negative or no cooperativity in the second half of the curve.

These obvious functional differences between the yeast and muscle enzymes are particularly interesting because of the high degree of homology in the primary structures of GPDHs from different species (Harris and Perham, 1968). One possible explanation for the functional differences is that the NAD

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[1] Abbreviations used are: GPDH, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); ADP-ribose, adenosine diphosphoribose; acetylpyridine-AD, acetylpyridine-adenine dinucleotide; thionicotinamide-AD, thionicotinamide–adenine dinucleotide; deamino-NAD, nicotinamide–hypoxanthine dinucleotide; deaminoacetylpyridine-AD, acetylpyridine–hypoxanthine dinucleotide; DTT, dithiothreitol; IAA, iodoacetic acid.

binding site of the yeast enzyme might interact with the coenzyme quite differently than does the binding site of the muscle enzyme. In order to test this possibility we have examined the binding of several coenzyme analogs to muscle GPD; Stockell (1959) has previously reported similar studies for the yeast enzyme. The data reported in this paper show that the differences in binding of the analogs to the two enzymes are small and cannot account for the functional dissimilarity of these enzymes.

From the analog studies with GPDH together with the recent reports on the preferred conformations of NAD and its analogs (Sarma and Kaplan, 1968, 1970a,b; Sarma *et al.*, 1968, 1970), it has been possible to draw some conclusions about the contact points between GPDH and NAD and about the probable conformation of NAD when it is bound to the enzyme. This structure is discussed and compared to the known structure of the lactate dehydrogenase–NAD complex (Adams *et al.*, 1969, 1970).

Experimental Section

Materials. Crystallized rabbit muscle glyceraldehyde 3-phosphate dehydrogenase was purchased as an ammonium sulfate suspension from Sigma Chemical Co. Glyceraldehyde 3-phosphate was obtained as the DL-glyceraldehyde 3-phosphate diethyl acetal barium salt from Sigma. The compound was deionized on Dowex 50-X4 and the resulting solution heated to 100° for 3 min to obtain the free aldehyde. β-NAD and NAD analogs were also from Sigma. NAD analogs included: α-NAD, NADP, AMP, ADP, ATP, NMN, ADP-ribose, acetylpyridine-AD, thionicotinamide-AD, deamino-NAD, deamino-NADP, deaminoacetylpyridine-AD. Dithiothreitol was from Calbiochem. All other reagents were analytical grade.

Charcoal Treatment of GPDH. A solution of the enzyme (about 3 mg/ml) in 0.01 M phosphate–1 mM EDTA–1 mM DTT (pH 7.0) was treated with 3–4 mg of activated charcoal/mg of protein (Velick, 1953). This produced apoenzyme with A_{280}/A_{260} of 1.8–1.9 and with about 90% of the activity of untreated enzyme. Based on the results of Fox and Dandliker (1965) this absorbance ratio represents 0.15–0.2 equiv of NAD bound per equiv of enzyme. The molecular weight of GPDH was taken to be 140,000 (Harris and Perham, 1965). The protein concentration of apoenzyme and holoenzyme was determined using the 280-mμ extinction coefficients of Murdock and Koeppe (1964), 0.815 and 1.06 $cm^2\ mg^{-1}$ for apo- and holoenzymes, respectively.

Enzymatic Assays. GPDH activity was measured with a Zeiss PMQ II spectrophotometer according to a modification of the standard method (Velick, 1955). The assay mixture contained 2.0 mM β-NAD, 2.0 mM DL-glyceraldehyde 3-phosphate, 10 mM sodium arsenate, 10 mM EDTA, and 0.066 M sodium pyrophosphate with a final pH of 8.0 in a total volume of 1.0 ml. Assays were initiated by addition of 10 μl of the enzyme and the increase in absorbance at 340 mμ was followed at 10-sec intervals to obtain the initial rate. The specific activity of GPDH was expressed as micromoles per minute per milligram of protein.

Coenzyme Analog Substrate Activity. The standard enzyme assay was used except that the assay mixture was made 2 mM with respect to the NAD analog rather than β-NAD. Substrate activity was expressed as per cent activity in the reduction reaction relative to β-NAD activity at the same concentration. K_m and V_{max} were determined from double-reciprocal plots.

Inhibitor Constants. Inhibition studies were carried out under the conditions of the standard assays but with NAD concentrations in the range of 4×10^{-5} to 4×10^{-4} M or glyceraldehyde 3-phosphate concentrations in the range of 7×10^{-5} to 7×10^{-4} M and inhibitor concentrations above and below the K_i values. In all cases at least five concentrations of substrate and four concentrations of inhibitor were used. Lineweaver–Burk and Dixon plots from experiments that employed varying substrate concentrations performed at several different inhibitor concentrations were used to determine K_i values.

Equilibrium Dialysis. Dialyses were carried out in 1.0-ml cells for 24 hr at 4° with gentle shaking. Controls showed that equilibrium was reached in that time. Ligand concentration on both sides of the membrane was determined from the extinction coefficient at the maximum absorbance wavelength for the analog. About 1.8 mg/ml of apoenzyme was dialyzed against NAD analog in 0.01 M phosphate–1 mM EDTA–1 mM DTT (pH 7.0) buffer. The λ_{max} and corresponding extinction coefficient for the analogs used are: β-NAD, 17,800 at 259 mμ; thinicotinamide-AD, 19,700 at 259 mμ (Seigel *et al.*, 1959; Anderson *et al.*, 1963).

Spectrophotometric Titration. Dialyzed solutions of apoenzyme (1.0–2.0 mg/ml) in 0.01 M phosphate–1 mM EDTA–1 mM DTT (pH 7.0) were scanned from 500 to 300 mμ in a Cary 14 spectrophotometer using the 0–0.1 slide-wire. Absorbance at 360 mμ was measured as a function of added NAD analog, and absorbance of the free analog was compensated for by adding equal aliquots of analog to the reference cuvet. The concentration of analog required to saturate the total GPDH sites was determined graphically from a plot of absorbance at 360 mμ *vs.* concentration of added analog. The per cent saturation at lower analog concentrations was calculated as the ratio of the 360-mμ absorbance at these levels to the absorbance at saturation.

Thermal Stability. The thermal stability of charcoal-treated GPDH was determined by incubating 1.0 ml of the enzyme (0.2–0.3 mg/ml) in 0.01 M phosphate–1 mM DTT–1 mM EDTA (pH 7.0) at 45° and periodically assaying 10-μl aliquots for enzyme activity. The first-order inactivation rate constants were obtained from a semilog plot of per cent initial activity *vs.* time of incubation in the presence or absence of 100 μM analogs. Each GPDH–analog rate constant was compared with that obtained simultaneously for the pure apoenzyme prepared for the particular experiment and the results were expressed as rate constant ratios k(analog)/k(no analog).

Results

Substrate Activity. Of the 13 analogs tested, only 3 were effective as substrates in the oxidation of phosphoglyceraldehyde under standard assay conditions: acetylpyridine-AD, thionicotinamide-AD, and deamino-NAD. The values for K_m and V_{max} for these analogs are shown in Table I. The three analogs which are effective substrates gave normal Michaelis–Menten kinetics and showed no evidence of cooperative or anticooperative behavior under the conditions used in these assays.

Two other analogs, acetylpyridinedeamino-AD and NADP, showed slight activity with the enzyme. At a concentration of 2 mM both gave 1% of the activity of NAD when assayed with the apoenzyme but there was no activity with the holoenzyme.

Inhibition. Several other analogs are inhibitors of the reaction and in all cases these are competitive with NAD. The K_i values are given in Table II. In the group of analogs which

TABLE I: Kinetic and Binding Constants for NAD Analogs as Substrates for GPDH.[a]

Analog	K_m (μM)	Rel V_{max}	K_d (μM)
β-NAD	50	1.0	16
Acetylpyridine-AD	500	0.1	
Thionicotinamide-AD	310	0.2	98
Deamino-NAD	2300	0.8	112

[a] The V_{max} values are the ratios of V_m(analog) : V_m(β-NAD). The following analogs gave no activity: pyridinecarbaldehyde-AD, NMN, NMN + AMP, α-NAD, deamino-NADP. The activity observed with NADP and acetylpyridinedeamino-NAD was too low and required too high concentrations to permit accurate determinations of K_m and V_{max}.

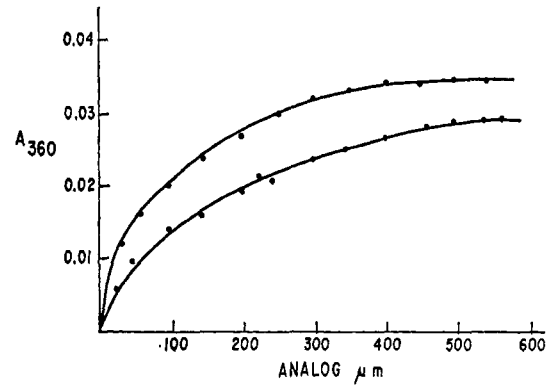

FIGURE 1: Titration of charcoal-treated rabbit muscle GPDH with NAD analogs. Charcoal-treated GPDH, A_{280}:A_{260} = 1.80, was titrated to maximum absorbance at 360 mμ. Enzyme concentration was 14 μM with a specific activity of 28. Enzyme and analogs were dissolved in 0.01 M phosphate–1 mM EDTA–1 mM DTT (pH 7.6).

TABLE II: Inhibitor Constants for NAD Analogs.[a]

Analog	K_i (mM)
Pyridinecarbaldehyde-AD	0.025
ADP-ribose	0.18
AMP	2.5
AMP + NMN	2.5
NADP	2.7
NMN	2.8
ADP	2.9

[a] The other analogs which were not active as substrates (α-NAD, deamino-NADP, and acetylpyridinedeamino-NAD) were not inhibitors.

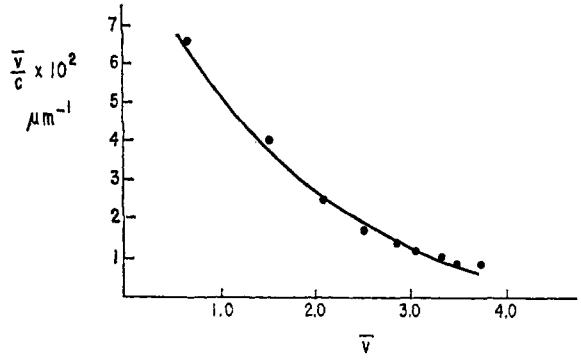

FIGURE 2: Scatchard plot for the binding of deamino-NAD to rabbit muscle GPDH. Four independent and equivalent binding sites for the analog were assumed in the calculation but the curvature of the plot indicates that there is anticooperativity in the binding of this analog to multiple sites.

represent partial structures of NAD the most effective inhibitor is ADP-ribose. The mixture of AMP + NMN contains all the moieties of NAD except the pyrophosphate bond and this mixture is no more inhibitory than AMP alone.

By far the most effective inhibitor of all the analogs tested is pyridinecarbaldehyde-AD. This analog is unusual in that it is competitive not only with NAD but also with the substrate phosphoglyceraldehyde. NADP is a weak inhibitor competitive with NAD. All other analogs which were not substrates gave no inhibition when tested at concentrations up to 4 mM.

Equilibrium Dialysis Studies. The values for the dissociation constants for β-NAD, thionicotinamide-AD, and deamino-NAD as measured by equilibrium dialysis are given in Table I. In order to reach equilibrium it was necessary to carry out the experiments for at least 18 hr. β-NAD, thionicotinamide-AD, and deamino-NAD were stable over this period but it was not possible to use acetylpyridine-AD in these experiments. Over a period of several hours at either 25 or 4° aqueous solutions of this analog developed a yellow color and gave a progressive increase in absorbance at 260 mμ. The value obtained for β-NAD is comparable to that reported by Velick (1958) and the value of K_i obtained by Conway and Koshland (1968).[2] The K_d for thionicotinamide-AD at 4° is about one-third that of its K_m. Deamino-NAD is quite different from β-NAD or thionicotinamide AD in that its K_m is at least 20 times higher than the K_d measured by equilibrium dialysis.

Spectrophotometric Properties of the Enzyme Analog Complexes. Racker and Krimsky (1952) first showed that the binding of β-NAD to the enzyme produces a broad absorbance band with a maximum at 360 mμ. Figure 1 shows the increase in absorbance at 360 mμ produced by successive additions of the two analogs which gave this effect. From these data it is possible to obtain values of per cent saturation as a function of the concentration of added analog. Although there are known to be four binding sites for NAD and one can assume that there are also four binding sites for the analogs, these data do not define how many of the actual binding sites contribute to the absorbance at 360 mμ.

When the data for deamino-NAD were calculated on the basis of four binding sites and the data plotted on the Scatchard plot the curve shown in Figure 2 was obtained. The marked curvature of the plot indicates that if all four sites are contributing to the absorbance the binding constants are not identical. The corresponding data obtained for acetylpyridine-NAD are shown in Figure 3. When the same data were recalculated assuming one, two, or three effective binding sites, similar sharp curvatures in the Scatchard plots were obtained. Treatment of the data for four sites by the method of Conway and Koshland (1968) to obtain the dissociation constant for

[2] The conditions used by us in this experiment and by Velick were such that only K_i is measured.

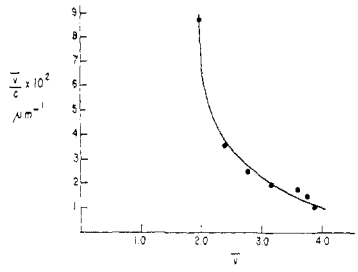

FIGURE 3: Scatchard plot for the binding of acetylpyridine-AD to rabbit muscle GPDH.

the final site failed to produce a linear Scatchard plot. Because of the uncertainty of the number of binding sites which produce the measured spectral change one can say with certainty only that there is more than one of these and they are not independent and identical. If one assumes four binding sites and calculates the average K_d between 50 and 80% saturation, a value of about 40 μM is obtained and the site or sites at lower per cent saturation must have K_d at least five times lower than this.

Thermal Stability of the Enzyme–Analog Complexes. The apoenzyme of GPDH is markedly unstable at neutral pH both at 4° and at elevated temperatures while the enzyme with bound NAD is stable under these conditions (Grisolia and Joyce, 1959). Each of the analogs was tested to determine whether it affected the thermal stability of the apoenzyme. It was found that incubation of the apoenzyme under the conditions shown in Figure 4 gave a first-order decay in enzyme activity. Each analog was tested at a concentration of 100 μM for its effect on the stability of the enzyme and the loss of activity was determined as a function of time. When appropriate, other concentrations of the analogs were employed as well. In all cases the loss in enzyme activity followed first-order kinetics and values of the rate constants were obtained. The reported values in Table III are the ratios of these rate constants in the presence and absence of the analog. Under the conditions used there was no loss of enzyme activity when the enzyme was saturated with β-NAD and consequently the ratio for β-NAD is zero. For analogs which give no protection against inactivation the ratio is 1.0.

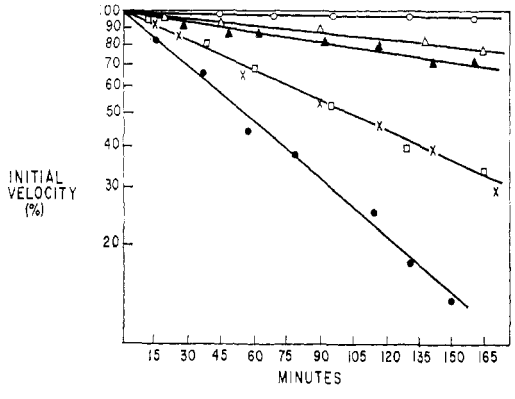

FIGURE 4: Effect of NAD and NAD analogs on the stability of GPDH at 45°. Protein concentration is 0.3 mg/ml in 0.01 M phosphate–1 mM EDTA (pH 7.0). The charcoal-treated protein was incubated at 45° and aliquots were removed for assay under the standard conditions at the indicated times. The additions were: (●) none, (○) 100 μM NAD, (△) 100 μM ADP-ribose, (▲) 100 μM thionicotinamide-AD, (×) 100 μM NMN, and (□) 100 μM ADP.

TABLE III: Effect of NAD Analogs on Thermal Inactivation of GPDH.[a]

Analog	Rate Constant Ratio k(analog)/k(no analog)
β-NAD	0
ADP-ribose	0.17
Thionicotinamide-AD	0.25
ADP	0.50
NMN	0.50
AMP + NMN	0.50

[a] The concentration of apoenzyme was 0.3 mg/ml and all analogs were 0.1 mM. The analogs which failed to stabilize the enzyme were: deamino-NAD, acetylpyridine-AD, pyridinecarbaldehyde-AD, AMP, NADP, α-NAD, deamino-NADP, and acetylpyridinedeamino-AD.

The analogs most effective in stabilizing the enzyme were ADP-ribose and thionicotinamide-AD. ADP afforded partial protection as did NMN.[3] AMP did not stabilize the enzyme and the mixture NMN + AMP was no more effective than NMN alone. All other analogs tested (Table III) failed to stabilize the enzyme even when used at concentrations sixfold higher than those reported here.

Discussion

Comparisons to Yeast GPDH. Although the GPDH from yeast and muscle are similar in many respects, it has been shown that they differ in one important function, namely, the yeast enzyme exhibits cooperative binding of NAD while the muscle enzyme shows anticooperative binding of the coenzyme (Cook and Koshland, 1970; Kirschner *et al.*, 1966; Conway and Koshland, 1968). It was thought that a comparison of the two enzymes using NAD analogs might provide some insight into this functional difference; however the date summarized in Table IV shows that the two enzymes are very similar at least with respect to the three parameters for which comparable data are available.

Both enzymes produce an absorbance band at 360 mμ when complexed with β-NAD or with two analogs which are effective substrates. The values of the dissociation constants for the analogs and β-NAD are very similar for the two enzymes. It should be emphasized that these values, as obtained by equilibrium dialysis or inhibition studies (muscle enzyme) or by spectrophotometric titration (yeast enzyme), are the binding constants for the final binding site of both enzymes. Because of the anticooperative binding of the muscle enzyme the $K_{dissoen}$ for the first binding site is several orders of magnitude lower than is that of the yeast enzyme. In the spectrophotometric titration studies carried out with the muscle enzyme we have shown that there is anticooperative binding of the two analogs which are effective substrates but that the

[3] Although it is clear that NMN binds to the muscle enzyme there is a report that NMN does not inhibit the yeast GPDH (Yang and Deal, 1969). This difference is not reflected in the results with any of the other analogs and remains an anomaly.

TABLE IV: Comparison of the NAD Analogs to Yeast and Muscle GPDH.[a]

Analog	Absorbance of Complex at 360 mμ		$K_{dissocn}$		Rel V_{max}	
	Muscle	Yeast	Muscle	Yeast	Muscle	Yeast
β-NAD	Yes	Yes	16 μM	7.8 μM	100	100
Acetylpyridine-AD	Yes	Yes	500	260	10	8.7
Deamino-NAD	Yes	Yes	112	130	80	8.3
Pyridinecarbaldehyde-AD	No	No	25	13	0	0
Acetylpyridinedeamino-AD	No	No		4800	1	1.7
Thionicotinamide-AD			98		20	

[a] The data for the yeast enzyme are those reported by Stockell (1959).

binding to the final site has a $K_{dissocn}$ similar to that of the yeast enzyme. The only obvious discrepancy between the results with the yeast and the muscle enzymes is the V_{max} value for deamino-NAD. In all other respects this analog gives similar results with the two enzymes.

From these results it appears that there is no significant qualitative difference between the coenzyme binding sites for the yeast and muscle enzymes sufficient to account for the functional difference between cooperative and anticooperative binding in the two cases. Thus it appears that the cooperative or anticooperative binding phenomena are related to differences in the structure or conformation of the enzymes outside the immediate binding sites for the coenzymes.

Important Interaction Sites between NAD and Rabbit Muscle GPDH. Another result of these studies in the finding that there is an important interaction of the ribose phosphate moiety of the NMN half of the coenzyme with the NAD binding site. For example, the dissociation constant for ADP ribose is at least ten times lower than that of ADP or AMP. The direct interaction of the phosphate of the NMN half of the coenzyme is suggested by the fact that both NMN and ADP produce some stabilization of the enzyme while AMP does not. This stabilization effect is in part synergistic with the binding of both the adjacent covalently bound phosphate on one side and the covalently bound ribose on the other. When both adjacent residues are present, as in ADP-ribose, the resulting complex has stability approaching that of the β-NAD complex and greatly in excess of the complexes of any one of the smaller analogs or of the mixture of NMN + AMP.

Previous reports have concluded that the principal form of β-NAD in aqueous solution at neutral pH has the aromatic rings stacked (Sarma and Kaplan, 1968, 1970a; Sarma *et al.*, 1970) and that the reduced coenzyme when bound to GPDH is in a conformation with the aromatic rings stacked (Velick, 1958, 1961). Although these points are still open to some criticism (*cf.* Jacobus, 1970), the data obtained with the NAD analogs are consistent with the binding of β-NAD in a stacked conformation with a strong interaction with the enzyme at the ribose–phosphate moiety of the NMN half of the coenzyme. Of the three analogs which are effective substrates, only thionicotinamide-AD partially stabilizes the enzyme. According to Sarma and Kaplan (1968, 1970a) acetylpyridine-AD exists in a stacked conformation, but, unlike β-NAD, the acetylpyridine ring is in the anti rather than the syn conformation with resulting changes in the conformation of the PPR backbone. Deamino-NAD occurs primarily in the open conformation (Sarma and Kaplan, 1968, 1970a). The unusually high K_m for this analog and its failure to stabilize the enzyme suggest that only the stacked conformation is active as a substrate.

In trying to obtain an approximate picture of the NAD binding site we have used the molecular model of the stacked form of the coenzyme in the P helix.[4] The model shown schemactically in Figure 5 is consistent with all data which are presently available. It is notable that in this orientation of the P helix the substituents of the ribose ring attached to nicotinamide moiety project back into the proposed site and the ionizable groups of the pyrophosphate backbone project to the left and back of the site.

Comparisons to the Coenzyme Binding Site of Lactate Dehydrogenase. There are some clear distinctions which can be made between the coenzyme binding site of GPDH and that of lactate dehydrogenase. The binding of AMP to lactate dehydrogenase is a prerequisite to the binding of NMN (McPherson, 1970), in contrast with our results with GPDH. According to McPherson the space group change of crystalline lactate dehydrogenase produced by a mixture of NMN + AMP differs from that produced by AMP alone and there is no change produced by NMN alone. We have observed no synergistic effects of NMN + AMP on the properties of GPDH but we do find effects of NMN or AMP alone.

Velick (1958, 1961) in his fluorescence studies of lactate dehydrogenase and GPDH concluded that the coenzyme binds in an open conformation to lactate dehydrogenase and in a stacked conformation to GPDH and the crystallographic data of Adams *et al.* (1969, 1970) clearly confirm the first of these conclusions. It therefore appears that all data so far available are consistent with the conclusion of Velick that lactate dehydrogenase and GPDH are representatives of two different classes of dehydrogenases, one which binds NAD in an open conformation and the other which binds NAD in a stacked conformation.

[4] The reason for choosing the P helix rather than the M is as follows. It has been shown that in the GPDH reaction the H is introduced from the substrate onto the "B" face of the nicotinamide ring (Vennesland and Westheimer, 1954; Levy *et al.*, 1962). The formation of an apparent charge transfer complex with tryptophan in the enzyme–NAD complex suggests that the exposed face of the nicotinamide ring is close to a tryptophan residue and, furthermore, there is evidence that the electron transfer in some dehydrogenases may be mediated by tryptophan (Schellenberg, 1967; Chan and Schellenberg, 1968; but see also Allison *et al.*, 1969). The "B" face of the nicotinamide ring is exposed in the P helix but not the M helix of β-NAD and therefore we propose that the bound conformation of β-NAD is that of the P helix.

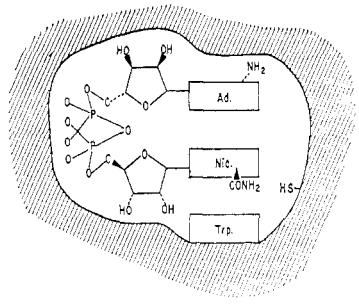

FIGURE 5: Schematic view of β-NAD at the active site of GPDH. The enzyme is in contact with the molecule on all sides except the one facing out of the page. The pyridine ring is stacked above a tryptophan residue and in the P-helical form the amide side chain is directed toward the front of the site. A cysteine sulfhydryl group to the right of the tryptophan residue would be accessible to the substrate and the C_4 position of the pyridine ring as well as to various sulfhydryl reagents. The ribose ring attached to pyridine in this P-helical form is below and to the left of the pyridine ring and both OH groups project to the left and back of the molecule. Both phosphate residues are above these OH groups with one almost directly in back of the other. All the oxygen substituents of the phosphate groups except the bridge oxygen are directed either to the left or top of the molecule and, together with the ribose substituents, provide a compact region where there appears to be major contact with the site. The ribose attached to adenine projects forward and its two OH groups are directly in front of the molecule. Phosphate substituents on either of these positions would have to be positioned above the rest of the molecule and thus could hinder binding due either to steric hindrance or to electrostatic repulsion with groups on the protein. The adenine ring is stacked above the pyridine ring and in this P helix the amino group is directly behind the molecule where it would also be buried in the complex.

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