Covalent Binding of 3-Pyridinealdehyde Nicotinamide Adenine Dinucleotide and Substrate to Glyceraldehyde 3-Phosphate Dehydrogenase*

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SUMMARY

Glyceraldehyde 3-phosphate dehydrogenase (α-glyceraldehyde-3-phosphate:nicotinamide adenine dinucleotide oxidoreductase; phosphorylating, EC 1.2.1.12) forms a complex with 3-pyridinealdehyde-NAD which survives precipitation with 7% perchloric acid. The molar ratio of bound 3-pyridinealdehyde-NAD to the enzyme is 2.5 to 2.9. Lactate, malate, and alcohol dehydrogenases do not form acid-p precipitable complexes with 3-pyridinealdehyde-NAD. 3-Pyridinealdehyde-deamino-NAD or glyceraldehyde 3-phosphate also forms an acid-stable complex with glyceraldehyde 3-phosphate dehydrogenase; however, NAD, 3-acetylpyridine-NAD, or thionicotinamide-NAD does not produce an acid-stable complex.

Incubation of the glyceraldehyde 3-phosphate dehydrogenase with glyceraldehyde 3-phosphate, acetyl phosphate, iodoacetic acid, or iodosobenzoate inhibits the formation of the acid-stable complex with 3-pyridinealdehyde-NAD. Glyceraldehyde 3-phosphate or 3-pyridinealdehyde-NAD also prevents carboxymethylation of the active site cysteine-149 by [14C]iodoacetic acid. These studies indicate that the aldehyde group of 3-pyridinealdehyde-NAD forms a thiohemiacetal linkage with cysteine-149 which is the substrate binding site for the dehydrogenase reaction. These findings may account for the fact that 3-pyridinealdehyde-NAD strongly inhibits the dehydrogenase and esterase activities of glyceraldehyde 3-phosphate dehydrogenase which require reduced cysteine-149. However, the analogue does not inhibit the acetylphosphatase activity of the enzyme for which the active site sulphydryl residues must be oxidized.

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is known to bind the coenzyme NAD approximately 1000 times more tightly than do other dehydrogenases (1). The rabbit muscle enzyme crystallizes with 3 to 4 mol of NAD bound to each mole of enzyme (2, 3). This enzyme-coenzyme complex is not dissociated by dialysis (4) or by passing it through Sephadex columns (5). However, there are several reasons for believing the coenzyme is not bound covalently to the protein. First, charcoal treatment of the enzyme complex can remove the coenzyme (4). In addition, [32P]NAD has been shown to exchange with bound NAD (6). Finally, when the conformation of the binding site is disturbed by acid precipitation, the enzyme-coenzyme complex dissociates.

In this paper, the nature of the NAD binding interactions is investigated by the use of NAD analogues. Specific modifications of the NAD molecule give clues to the importance of particular atoms in NAD binding and the reactivity of the active site of this enzyme. In particular, the NAD analogue 3-pyridinealdehyde-NAD produces an acid-stable enzyme-coenzyme complex. The substrate, glyceraldehyde 3-phosphate, also forms an acid-stable enzyme-substrate complex. Both of these complexes prevent carboxymethylation of cysteine-149 in the active site of this enzyme, suggesting that each of these compounds forms a thiohemiacetal bond in the active site. The nature of the 3-pyridinealdehyde-NAD complex has been characterized, and the effects of this analogue on the dehydrogenase, esterase, and acetylphosphatase activities of this enzyme have been investigated.

MATERIALS AND METHODS

Enzymes and Proteins—Glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle was recrystallized at least three times in the presence of 1 mM EDTA by the method of Cori et al. (2). Enzyme-bound NAD was removed with activated charcoal (5) and the apoenzyme was dialyzed for at least 2½ hours against 5 mM Tris-1 mM EDTA buffer, pH 6.8, in order to remove ammonium sulfate. The apoenzyme gave a ratio of optical density readings at 280 and 360 nm which varied from 1.75 to 1.9. The enzyme concentration was determined from the extinction coefficient at 280 nm (2) and the molecular weight of the enzyme was taken as 140,000 (3).

Malate dehydrogenase was obtained from Boehringer Mannheim. Alcohol dehydrogenase was purchased from Worthington Biochemical Corp. Lactate dehydrogenase, aldolase, and papain were purchased from Sigma Chemical Co. Bovine albumin was obtained from Armour Laboratories.

Substrates, Coenzymes, NAD Analogues, and Inhibitors—DL-Glyceraldehyde 3-phosphoric acid diethylacetal, barium salt, was purchased from Calbiochem and was prepared according to the supplier's instructions. NAD and NADH were obtained from Sigma. NAD analogues, 3-acetylpyridine-NAD, thionicotinamide-NAD, and 3-acetylpyridine-NAD deamino-NAD do not produce an acid-stable complex.
tinamide-NAD, 3-pyridinealdehyde-NAD, and 3-pyridinealdehyde-deamino-NAD, were purchased from P-L Biochemicals. Acetyl phosphate was prepared by the procedure of Stadtman and Lipmann (7). Iodosobenzoate was obtained from Sigma and sodium tetraorthotinate from K & K Laboratories. [14C]iodoacetic acid was obtained from New England Nuclear and was purified by recrystallization from carbon tetrachloride. The specific activity of the [14C]iodoacetic acid was 3.36 × 10^6 cpm μmol⁻¹. Unlabeled iodoacetic acid was obtained from Mann Research Laboratories.

**Analytical Methods.** The enzyme-coenzyme complex was formed by incubating the enzyme with NAD or an NAD analogue for 15 min at 0° in 0.1 M Tris buffer, pH 7.0. The protein was precipitated with 7% (v/v) perchloric acid, and the amount of bound coenzyme was determined by two methods. First, the optical density of the supernatant solution was measured spectrophotometrically at 260 nm in order to determine the amount of coenzyme removed from the reaction mixture. The optical density of the supernatant at 260 nm was corrected for the reading of the protein alone after acid precipitation. In the second technique, the phosphorus content of the precipitated protein was determined by the method of Mann Research Laboratories.

The precipitate was washed three times in 2.0 ml of 7%, perchloric acid until no optical density at 260 nm was detected. After washing, the precipitate was washed, and the total phosphate content was determined by a Technicon AutoAnalyzer by the method of Yee (8). Weak binding of coenzymes to the apoenzymes was measured by filtering the complexes through an Amicon Diaflo ultrafiltration membrane (PM-30) as described in Table VI. The procedure for carboxymethylating glyceraldehyde 3-phosphate dehydrogenase with [14C]iodoacetic acid was carried out as follows: dialyzed apoenzyme (14 mg; 0.10 pmol) was incubated with [14C]iodoacetic acid (2.0 μmol) in 0.1 M Tris-1 mM EDTA buffer, pH 7.16, at 0° for 20 min in a total volume of 2.1 ml. The reaction was stopped by the addition of 7 ml of acetone-ether-HCl (1:8, 20:3:1, v:v:v). The precipitate was washed three times with 5 ml of acetone-ether-HCl and twice with acetone. The precipitate was dried thoroughly, and a weighed amount (approximately 5 mg) was suspended in 0.33 ml of 0.04 N HCl. The sample was digested with 0.3 ml of pepsin (1.0 mg per ml) for 1 hour. The amount of carboxymethylation was determined in a Packard 3375 liquid scintillation counter.

The assay procedures for the dehydrogenase and acetyl phosphate activities of glyceraldehyde 3-phosphate dehydrogenase are described in Fig. 2 and Table VII, respectively.

**RESULTS**

**Acid-stable Complexes between Glyceraldehyde 3-Phosphate Dehydrogenase and NAD Analogues or Substrate.** The stoichiometry of the binding of NAD analogues and substrate to glyceraldehyde 3-phosphate dehydrogenase was determined both spectrophotometrically and chemically (Table I). The spectrophotometric method gave slightly higher values for bound coenzyme, possibly because of the loss of precipitate during the washing procedure. 3-Pyridinealdehyde-NAD, 3-pyridinealdehyde-deamino-NAD, and the substrate, glyceraldehyde 3-phosphate, all formed acid-stable complexes with this enzyme. 3-Pyridinealdehyde-NAD had a higher binding ratio than 3-pyridinealdehyde-deamino-NAD. The exact stoichiometry of 3-pyridinealdehyde-NAD binding varied from 2.5 to 2.9 sites per molecule of enzyme, depending on the enzyme preparation and the completeness of the NAD removal by charcoal treatment. The enzyme which had been purified on a Sephadex G-100 column consistently gave a ratio near 2.9. The substrate binding could be determined only by the phosphate method, and 1.1 mol per mol of enzyme were bound. NAD, 3-acetylpyridine-NAD, and thioctic acid-NAD did not form acid-stable complexes with glyceraldehyde 3-phosphate dehydrogenase.

In order to investigate the specificity of this covalent binding of 3-pyridinealdehyde-NAD, seven other proteins were examined.

**TABLE I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Determined spectrophotometrically from supernatant</th>
<th>Determined as phosphate in precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Pyridinealdehyde-NAD</td>
<td>4.0</td>
<td>2.5</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-deamino-NAD</td>
<td>4.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>6.0</td>
<td>1.9</td>
</tr>
<tr>
<td>NAD</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>3-Acetylpyridine-NAD</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>Thioctic acid-NAD</td>
<td>4.0</td>
<td>0</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Moles of 3-pyridinealdehyde-NAD per mole of enzyme</th>
<th>Bound to enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>4.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>Yeast alcohol dehydrogenase</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>Liver alcohol dehydrogenase</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c malate dehydrogenase</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>Papain</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>Aldolase</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

In this study, four dehydrogenases, the glycolytic enzyme aldolase, the sulfhydryl protease papain, and serum albumin were selected. None of these proteins showed any 3-pyridinealdehyde-NAD binding after acid precipitation.

**Effects of Sulfonyl Hydrazide and Acetyl Phosphate on 3-Pyridinealdehyde-NAD Binding.** Sodium tetraorthotinate has been shown to react specifically with cysteine residues in the active site of muscle glyceraldehyde 3-phosphate dehydrogenase (10, 11). Preincubation of the enzyme with 4 eq of sodium tetraorthotinate completely inhibited 3-pyridinealdehyde-NAD binding (Table III). The effects of other sulfonyl hydrazides, iodoacetamide and iodosobenzoxazole, were investigated.
Effect of sodium tetrathionate on 3-pyridinealdehyde-NAD binding

Dialyzed, NAD-free glyceraldehyde 3-phosphate dehydrogenase (0.03 μmol) was incubated with varying amounts of sodium tetrathionate in 0.1 M Tris buffer, pH 7.0, for 15 min at 0°. Then 0.1 μmol of 3-pyridinealdehyde-NAD was added and the incubation was continued for another 15 min. The total volume was 1.0 ml. The reaction was terminated by adding 2.0 ml of 7% perchloric acid and aliquots of the supernatant were measured spectrophotometrically at 260 nm to determine free coenzyme.

The reaction was continued for another 15 min. The total volume was 1.0 ml; 1.2 pmol of iodoacetic acid and iodosobenzoate and 1.0 ml of 3-pyridinealdehyde-NAD were used. The bound 3-pyridinealdehyde-NAD was determined spectrophotometrically as described in Table I.

<table>
<thead>
<tr>
<th>Sodium tetrathionate</th>
<th>3-Pyridinealdehyde-NAD binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>mol/mol enzyme</td>
<td>mol/mol enzyme</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Effect of pH on inhibition of 3-pyridinealdehyde-NAD binding by acetyl phosphate

Dialyzed, NAD-free glyceraldehyde 3-phosphate dehydrogenase (0.06 μmol) was incubated at room temperature for 20 min with or without acetyl phosphate as indicated in the table. The pH was maintained at pH 4.5 with 0.02 M acetate buffer and at pH 7.0 or 8.5 with 0.1 M Tris. Then 3-pyridinealdehyde-NAD was added and was incubated for 15 min at 0°. The binding ratio was determined spectrophotometrically at 260 nm as described under "Materials and Methods."

![Graph](http://www.jbc.org/)

Fig. 1. The effects of coenzymes and substrate on the carboxymethylation of cysteine-149 by [14C]iodoacetic acid ([14C]JAA). The time course of the carboxymethylation of glyceraldehyde 3-phosphate dehydrogenase by [14C]iodoacetic acid was determined as described under "Materials and Methods." The experimental conditions were as follows: dialyzed, NAD-free enzyme (0.10 μmol) and [14C]iodoacetic acid (2 μmol) were incubated in 1 M Tris buffer, pH 7.16, at 0° after a 20-min preincubation. ▲—▲, apo-enzyme; □—□, enzyme preincubated with NAD (1.0 μmol); ■—■, enzyme preincubated with NADH (1.0 μmol); ○—○, enzyme preincubated with glyceraldehyde 3-phosphate (1.0 μmol); ×—×, enzyme preincubated with 3-pyridinealdehyde-NAD (1.0 μmol).

4.5, the amount of added 3-pyridinealdehyde-NAD was lowered accordingly.

Effect of Coenzymes and Substrate on Carboxymethylation of Glyceraldehyde 3-Phosphate Dehydrogenase by [14C]Iodoacetic Acid—The rate of carboxymethylation of the active site cysteine-149 by [14C]iodoacetic acid in the presence of various ligands of the enzyme is shown in Fig. 1. The apoenzyme and the enzyme preincubated with NAD both showed a final value of approximately 3.0 mol of [14C]iodoacetic acid bound per mol of enzyme. The half-maximal alkylation time for the apoenzyme was 7 min. When the apoenzyme was preincubated with NAD, the well known (12, 15, 16) enhancement of reactivity with iodoacetic acid...
formed before the addition of the sulfhydryl inhibitors, the complex may be protected against dissociation. This protection was stronger in the case of 3-pyridinealdehyde-NAD where iodoacetic acid inhibited carboxymethylation, whereas 3-pyridinealdehyde-NAD did not. The half-maximal alkylation times were estimated at 50, 180, and 210 min, respectively, by extrapolation. The reaction was started by the addition of glyceraldehyde 3-phosphate, 0.4 mM. The rate of the reaction was determined by following the appearance of glyceraldehyde 3-phosphate at 340 nm with a Gilford recording spectrophotometer. The velocities during the first 15 s were measured on the control with no added 3-pyridinealdehyde-NAD and at 3-pyridinealdehyde-NAD concentrations of 0.1 μM (X--X) and 66 μM (▲--▲). The ordinate is plotted in units of (mmoles per min per mg enzyme)−1.

was evidenced by a reduction in the half-maximal alkylation time to less than 1 min. Preincubation with NADH, glyceraldehyde 3-phosphate, or 3-pyridinealdehyde-NAD inhibited the rate of carboxymethylation. The half-maximal alkylation times were estimated at 50, 180, and 210 min, respectively, by extrapolation.

Measurement of Noncovalent Coenzyme Binding by the Ultrafiltration Method—Inasmuch as ultrafiltration in the Amicon Diaflo apparatus does not destroy the three-dimensional structure of the coenzyme binding site, it can be used to measure noncovalent as well as covalent coenzyme binding. With this technique, the apoenzyme bound 3.0 or 3.7 mol of NAD or 3-pyridinealdehyde-NAD per mol of enzyme, respectively (Table VI). Preincubation of the apoenzyme with iodoacetic acid caused a 30% inhibition of the enzyme-coenzyme complex formation. Under the same conditions, iodosobenzoate produced approximately 50% inhibition of binding. If the enzyme-coenzyme complex was formed before the addition of the sulfhydryl inhibitors, the complex may be protected against dissociation. This protection was stronger in the case of 3-pyridinealdehyde-NAD where iodoacetic acid or iodosobenzoate produced only a 3% or 20% dissociation of the preformed complex, respectively. These data corroborate the results of the [14C]iodoacetic acid binding studies in which NAD enhanced carboxymethylation, whereas 3-pyridinealdehyde-NAD strongly inhibited carboxymethylation.

Effect of 3-Pyridinealdehyde-NAD on Kinetics of Dehydrogenase, Esterase, and Acetyl Phosphatase Reactions—Kaplan et al. (17, 18) have shown that 3-pyridinealdehyde-NAD is a strong inhibitor of the dehydrogenase activity in the enzyme from muscle. With a ratio of 3-pyridinealdehyde-NAD to NAD of 1:18, the dehydrogenase reaction is inhibited by more than 50%. This strong inhibition was investigated by a kinetic study. A double reciprocal plot using a variable NAD concentration (Fig. 2) indicated that 3-pyridinealdehyde-NAD is a competitive inhibitor with respect to the coenzyme. A double reciprocal plot with a varying glyceraldehyde 3-phosphate concentration was more complex (Fig. 3). As the 3-pyridinealdehyde-NAD concentration was increased, the K_m for the substrate and the maximal velocity both increased.

The esterase reaction which splits p-nitrophenyl acetate to acetic acid and p-nitrophenol has been demonstrated by Park et al. (19). The effect of 3-pyridinealdehyde-NAD on this reaction was analogous to the effect of NAD, that is, stoichiometric inhibition of the rate of esterolysis by blocking acetylation of the enzyme.

The phosphatase activity of this enzyme is known to require both the presence of coenzyme and the oxidation of the cysteine-149 residue. Inasmuch as 3-pyridinealdehyde-NAD may bind to cysteine-149, one might expect that 3-pyridinealdehyde-NAD could produce phosphatase activity without requiring the addition of an oxidizing agent. Table VII shows that an oxidizing agent such as iodosobenzoate was still required to produce significant phosphatase activity. Without iodosobenzoate, only negligible activity was elicited by the addition of either NAD or 3-pyridinealdehyde-NAD. Although the activity with iodosobenzoate was reduced somewhat, 3-pyridinealdehyde-NAD did partially satisfy the coenzyme requirement for the phosphatase reaction.

**DISCUSSION**

An acid-stable complex was formed between rabbit muscle glyceraldehyde 3-phosphate dehydrogenase and 3-pyridinealde-
concentration was 0.60 mM and the substrate concentration was varied as shown on the abscissa. The velocities of the reaction procedure were the same as those for Fig. 2 except that the NAD (X——X) and 66 1.\'M (A——A).

The reaction was started by the addition of acetyl phosphate, and hydrolysis was allowed to proceed for 10 min. The samples then were warmed to 25\(^\circ\)C. The reaction mixture for the phosphatase assay contained the following: NAD-free glyceraldehyde 3-phosphate dehydrogenase, 0.005 \(\mu\)mol; iodosobenzoate, 1 \(\mu\)mol; NAD or 3-pyridinealdehyde-NAD, 1 \(\mu\)mol; acetyl phosphate, 6.7 \(\mu\)mol; and Tris buffer, pH 7.5, 100 \(\mu\)mol. Total volume was 2 ml. The NAD-free enzyme was incubated with coenzymes and iodosobenzoate as indicated in the table for 10 min at 0\(^\circ\)C. The samples then were warmed to 25\(^\circ\)C. The reaction was started by the addition of acetyl phosphate, and hydrolysis was allowed to proceed for 10 min. The disappearance of acetyl phosphate was quantitatively determined by the method of Lipmann and Tuttle (9).

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### Table VII

**Inhibition of acetyl phosphatase activity of glyceraldehyde 3-phosphate dehydrogenase by 3-pyridinealdehyde-NAD**

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Ratio of enzyme to coenzyme to iodosobenzoate in assay</th>
<th>Acetyl phosphate hydrolysed</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>1:200:0</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-NAD</td>
<td>1:200:0</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>NAD</td>
<td>1:200:200</td>
<td>4.2</td>
<td>100</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-NAD</td>
<td>1:200:200</td>
<td>2.9</td>
<td>69</td>
</tr>
<tr>
<td>NAD</td>
<td>1:200:200</td>
<td>4.1</td>
<td>98</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-NAD</td>
<td>1:50:200</td>
<td>2.7</td>
<td>64</td>
</tr>
</tbody>
</table>

Fig. 3. 3-Pyridinealdehyde-NAD inhibition of glyceraldehyde 3-phosphate dehydrogenase as a function of the concentration of glyceraldehyde 3-phosphate (GAP). The reaction mixture and procedure were the same as those for Fig. 2 except that the NAD concentration was 0.60 mm and the substrate concentration was varied as shown on the abscissa. The velocities of the reaction procedure were the same as those for Fig. 2 except that the NAD (X——X) and 66 1.\'M (A——A).

The specificity of the 3-pyridinealdehyde-NAD covalent binding which was tested on a variety of proteins indicates that this complex is not an artifact due to trapping of the coenzyme or a general chemical reaction with an amino acid. This specificity is surprising for two reasons: first, because of the structural homology which is found between the different dehydrogenases; and second, because each of these dehydrogenases has a thiol residue which has been implicated as “essential” to the catalytic mechanism (23-27). The structural homology was first shown between lactate dehydrogenase (28) and malate dehydrogenase (29). These two dehydrogenases have a very similar poly peptide backbone conformation throughout the entire molecule. Recently, horse liver alcohol dehydrogenase (30) and lobster glyceraldehyde 3-phosphate dehydrogenase (31) both have been shown to have a similar tertiary structure in the NAD binding area of the molecule.

Inasmuch as the coenzyme binding regions of these dehydrogenase proteins have similar three-dimensional structures and the coenzyme molecule itself has a flexible conformation which allows it to conform to the protein binding site, the specificity of the inhibitory effect of 3-pyridinealdehyde-NAD must result from a difference in spatial location or reactivity of the “essential” thiol residues in these dehydrogenase molecules. It remains to be shown by peptide sequencing that 3-pyridinealdehyde-NAD does in fact bind to cysteine-149 in the rabbit muscle enzyme.

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is inactivated irreversibly by alkylating reagents such as iodoacetic acid which react with the active site cysteine residue (2, 13). It is reversibly inactivated by reagents which oxidize thiol residues, such as iodosobenzoate (32) and sodium tetrathionate (11, 13). Competitive binding studies with each of these compounds indicate that the site of covalent binding of the aldehyde group is the same as the substrate binding site, cysteine-149. Sodium tetrathionate stoichiometrically inhibits 3-pyridinealdehyde-NAD binding, as would be predicted if they were competing for the same binding site (Table III). Preincubation with iodoacetic acid or iodosobenzoate inhibited the binding of 3-pyridinealdehyde-NAD. Iodosobenzoate will destroy the preformed acid-stable complex, presumably by attacking the covalent bond, iodosobenzoate being a slightly stronger inhibitor under the conditions tested (Table IV). Longer incubation times or higher incubation temperatures increase the displacement effect.

The studies with \(^{14}C\)iodoacetic acid showed that there is a slow time-dependent release of 3-pyridinealdehyde-NAD with binding of \(^{14}C\)iodoacetic acid to cysteine-149 (Fig 1). The 3-pyridinealdehyde-NAD displaced (approximately 10%) was less than that in Table IV due to variation in experimental conditions. The inhibitory effect of 3-pyridinealdehyde-NAD on \(^{14}C\)iodoacetic acid binding was essentially equivalent to that of the substrate, suggesting a similar bond.
Acetyl phosphate has been shown to exhibit a pH-dependent acetylation of the rabbit muscle enzyme (5, 14). At pH 4.5 the acetyl group is predominately in a thioester linkage with cysteine-149. At pH 7.0 some acetyl groups bind to cysteine, whereas others bind to the ε-amino group of a reactive lysine. At pH 8.5 the acetyl group is almost entirely in an N-acetyl linkage. Mathew et al. (14) have shown that the site of acetylation influences NAD binding. Using a Sephadex G-25 column to separate free and bound NAD, they found 2.6 sites of NAD bound to the native enzyme or S-acetyl enzyme prepared with acetyl phosphate at pH 4.5; however, only 1.2 sites were bound to the N-acetyl enzyme formed with acetyl phosphate at pH 8.5. Thus, lysine-183 appears essential for maximal NAD binding. Under the same conditions, 3-pyridinealdehyde-NAD has been shown to form an acid-stable complex with 2.6 sites of 3-pyridinealdehyde-NAD bound to the N-acetylated enzyme. This indicates that the thiohemiacetal bond formed in 3-pyridinealdehyde-NAD is so strong that lysine-183 is not necessary for binding.

The kinetic studies provide evidence about the effect of 3-pyridinealdehyde-NAD on the dynamics of enzymatic catalysis. In the esterase reaction, 3-pyridinealdehyde-NAD and NAD both inhibited the initial step of acetylation of the enzyme by the substrate, p-nitrophenyl acetate. This suggests that both coenzymes bind at the same site and can sterically block the reaction of the large substrate with its phenyl ring. The phosphatase reaction also implies that 3-pyridinealdehyde-NAD binds at the coenzyme binding site because bound coenzyme is known to be required for the phosphatase activity.

The analogue inhibition of the dehydrogenase reaction is very strong and somewhat complex because 3-pyridinealdehyde-NAD affects both the NAD and the glyceraldehyde 3-phosphate binding sites. Presumably the adenine end of 3-pyridinealdehyde-NAD binds in the normal adenine binding site. The nicotinamide moiety positions itself so that the aldehyde group can react with cysteine-149, forming a thiohemiacetal bond where the substrate normally forms such a bond.

Crystallographic studies (35) as well as kinetic studies (36) indicate that it is the adenosine moiety of NAD which is important in coenzyme binding to the native conformation of dehydrogenase enzymes. The ultratranslation experiments (Table VI) corroborate these studies. Both NAD and 3-pyridinealdehyde-NAD bind to the native conformation of the enzyme. This should be expected because the adenosine portion of both molecules is the same and is bound to the same adenosine binding site. However, under each condition tested in Table VI, 3-pyridinealdehyde-NAD showed a higher binding ratio than NAD. This implicates a difference in binding for the nicotinamide portions of these molecules, with aldehyde-nicotinamide having a higher binding affinity than nicotinamide.

The studies with the ultratranslation method when combined with the acid precipitation data indicate that 3-pyridinealdehyde-NAD binds noncovalently to sites on the enzyme independent of the state of reduction of cysteine-149. If this cysteine residue is reduced, the aldehyde group of 3-pyridinealdehyde-NAD can form a thiohemiacetal linkage to the thiol residue at the substrate binding site. It is this covalent bond which produces the acid-stable enzyme-coenzyme complex.

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