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

(Received for publication, June 17, 1974)

EDWARD J. HILL, TA-HSU CHOU, MARIA C. SHIH, AND JANE HARTING PARK

From the Department of Physiology and Neuromuscular Disease Research Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

SUMMARY

Glyceraldehyde 3-phosphate dehydrogenase (n-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-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.

The nature of this NAD binding interactions is investigated by the use of NAD analogues. The 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 substrate of the 3-pyridinealdehyde-NAD complex has been characterized, and the effects of this analogue on the dehydrogenase, esterase, and acetyl phosphatase 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 260 nm which varied from 1.75 to 1.9. The enzyme concentration was determined from the extinction coefficient at 280 nm (3) 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, were purchased from Calbiochem.

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, [H]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.
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 tetraiodoacetate 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 X 10⁶ 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 then 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 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 analyzed. The precipitate was washed three times in 2.0 ml of 7% perchloric acid until no optical density at 260 nm was detected in the washing solution. The precipitate was hydrolyzed completely in 70% perchloric acid, and the total phosphate content was determined on a Technicon AutoAnalyzer by the method of Yue (8).

Weak binding of coenzymes to the apoenzyme 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 µmol) 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 (18:5:1 (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 0.33 N HCl. The sample was digested with 0.3 ml of pepsin (1.0 mg per ml) for 1 h. 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 a 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 1.9 mol per mol of enzyme were bound. NAD, 3-acytylpyridine-NAD, and thioicinamide-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

| Compound                  | Initial addition | Determined spectrophotometrically from supernatant | Determined as phosphate in precipitate |
|---------------------------|------------------|--------------------------------------------------|---------------------------------------|
| 3-Pyridinealdehyde-NAD    | 4.0              | 2.5                                              | 1.8                                   |
| 3-Pyridinealdehyde-deamino-NAD | 4.0               | 1.7                                              | 1.6                                   |
| Glyceraldehyde 3-phosphate.| 6.0              | 0                                                | 0                                     |
| NAD                       | 4.0              | 0                                                | 0                                     |
| 3-Acetylpyridine-NAD      | 4.0              | 0                                                | 0                                     |
| Thioicinamide-NAD         | 4.0              | 0                                                | 0                                     |

**Table I**

**Stoichiometry of acid-stable complexes formed with glyceraldehyde 3-phosphate dehydrogenase**

Dialyzed NAD-free glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (0.03 µmol) was incubated with NAD analogues, substrate, and NAD in 0.1 M Tris buffer, pH 7.0, for 15 min at 0°. The total volume was 1 ml. Reactions were stopped by adding 2.0 ml of 70% (v/v) perchloric acid. Aliquots of supernatant solutions were measured spectrophotometrically at 260 nm, and the bound coenzyme was determined as discussed under "Materials and Methods." The precipitate was washed, and the total phosphate concentration was determined after oxidation with 0.5 ml of 70% perchloric acid.

**Table II**

**Protein specificity of 3-pyridinealdehyde-NAD binding**

3-Pyridinealdehyde-NAD was incubated with selected proteins in 0.1 M Tris buffer, pH 7.0, for 15 min at 0°. The addition of 3-pyridinealdehyde-NAD was essentially stoichiometric on a monomer basis. The enzyme was precipitated with perchloric acid, and the coenzyme removed from the supernatant solution was ascertained spectrophotometrically as described in Table I.
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 °C. 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.

| Sodium tetrathionate | 3-Pyridinealdehyde-NAD binding |
|----------------------|--------------------------------|
| mol/mol enzyme       | mol/mol enzyme | % inhibition |
| 0                    | 2.0             | 0            |
| 1                    | 1.6             | 21           |
| 2                    | 1.1             | 44           |
| 3                    | 0.6             | 71           |
| 4                    | 0.0             | 100          |
| 10                   | 0.0             | 100          |

**TABLE IV**

Effects of sulfhydryl inhibitors on binding of 3-pyridinealdehyde-NAD

Dialyzed, NAD-free glyceraldehyde 3-phosphate dehydrogenase (0.03 μmol) was incubated in 0.1 μmol Tris buffer, pH 7.0, at 0 °C for 15 min after each addition as indicated below. The total volume was 1.0 ml; 1.2 μmol of iodoacetic acid and iodosobenzoate and 0.12 μmol of 3-pyridinealdehyde-NAD were used. The bound 3-pyridinealdehyde-NAD was determined spectrophotometrically as described in Table 1.

| First addition | Second addition | Moles of 3-pyridinealdehyde-NAD bound per mole of enzyme |
|----------------|-----------------|---------------------------------------------------------|
| 3-Pyridinealdehyde-NAD | None             | 2.5                                                     |
| Iodoacetic acid     | 3-Pyridinealdehyde-NAD | 0.1                                                   |
| Iodosobenzoate      | 3-Pyridinealdehyde-NAD | 0.05                                                   |
| 3-Pyridinealdehyde-NAD | Iodoacetic acid   | 1.8                                                     |
| 3-Pyridinealdehyde-NAD | Iodosobenzoate   | 0.8                                                     |

Also (Table IV). Iodoacetic acid carboxymethylates only the reactive cysteine-149 in the active site of this dehydrogenase, whereas iodosobenzoate reacts first with the active site cysteine and subsequently oxidizes other —SH residues (11-13). As shown in Table IV, when iodoacetic acid or iodosobenzoate was added first, the inhibitor completely prevented the covalent 3-pyridinealdehyde-NAD binding. However, when the 3-pyridinealdehyde-NAD was added first, iodoacetic acid displaced only 0.7 mol of the analogue, whereas iodosobenzoate removed 1.7 mol of the 2.5 mol of bound analogue.

In order to compare the analogue binding capacity of S-acetylated enzyme and N-acetylated enzyme, the pH dependence of acetyl phosphate inhibition of 3-pyridinealdehyde-NAD binding to the enzyme was investigated (Table V). Inhibitions of 65% and 25% were observed at pH 4.5 and 7.0, respectively, whereas no inhibition was found at pH 8.5. Thus, it can be concluded that N-acetylation does not block analogue binding because Park et al. (5, 14) have shown that at pH 4.5 an S-acetyl enzyme complex is formed with cysteine-149 and at pH 8.5 an N-acetyl enzyme complex is formed with lysine-185. Inasmuch as acetyl phosphate forms a complex with only two catalytic sites at pH 4.5, the amount of added 3-pyridinealdehyde-NAD was lowered accordingly.

**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 °C. The binding ratio was determined spectrophotometrically at 260 nm as described under "Materials and Methods."

| pH | Acetyl phosphate | 3-Pyridinealdehyde-NAD per mole of enzyme | Inhibition of 3-pyridinealdehyde-NAD binding |
|----|------------------|-----------------------------------------|---------------------------------------------|
|    | Added | Bound | %                          |
| 4.5 | None | 2.0 | 1.3 | 60 |
| 10  | 2.0 | 0.0 | 60 |
| 7.0 | None | 1.9 | 1.1 | 27 |
| 20  | 1.9 | 0.8 | 27 |
| 8.5 | None | 1.9 | 1.1 | 0  |
| 20  | 1.9 | 1.1 | 0  |

**FIG. 1.** The effects of coenzymes and substrate on the carboxymethylation of cysteine-149 by [³⁵Cl]iodoacetic acid ([³⁵Cl]IAA). The time course of the carboxymethylation of glyceraldehyde 3-phosphate dehydrogenase by [³⁵Cl]iodoacetic acid was determined as described under "Materials and Methods." The experimental conditions were as follows: dialyzed, NAD-free enzyme (0.10 μmol) and [³⁵Cl]iodoacetic acid (2 μmol) were incubated in 1 M Tris buffer, pH 7.16, at 0 °C 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).
TABLE VI

Binding of NAD or 3-pyridinealdehyde-NAD to native glyceraldehyde 3-phosphate dehydrogenase

In the ultrafiltration technique, 0.04 µmol of dialyzed apoenzyme was incubated as indicated below with 0.2 µmol of coenzyme (NAD or 3-pyridinealdehyde-NAD) and 0.2 µmol of inhibitor (iodoacetic acid or iodosobenzoate) in 0.1 M Tris buffer, pH 7.0. After each addition, the reaction mixture was incubated for 15 min at 0°C. At the end of the incubations, the solution was passed through an Amicon Diaflo ultrafiltration membrane PM-30 under a nitrogen pressure of 55 p.s.i. The membrane was washed three times with 3.0 ml of glass-distilled water in order to recover the enzyme-coenzyme complex. The filtrate and protein solutions were measured at 260 and 280 nm, and the amount of enzyme-coenzyme complex was estimated both as free and bound coenzyme.

| First addition | Second addition | Moles of coenzyme bound per mol of enzyme |
|----------------|-----------------|------------------------------------------|
| None           | NAD             | 3.0                                      |
| Iodoacetic acid| NAD             | 2.0                                      |
| Iodosobenzoate | NAD             | 1.7                                      |
| NAD            | Iodoacetic acid | 2.2                                      |
| NAD            | Iodosobenzoate  | 2.4                                      |
| None           | 3-Pyridinealdehyde-NAD | 3.7                                    |
| Iodoacetic acid| 3-Pyridinealdehyde-NAD | 2.3                                    |
| Iodosobenzoate | 3-Pyridinealdehyde-NAD | 1.7                                    |
| 3-Pyridinealdehyde-NAD | Iodoacetic acid | 3.6                                      |
| 3-Pyridinealdehyde-NAD | Iodosobenzoate | 3.1                                      |

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—As in the 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-pyridinealdehy-
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 glyceraldehyde 3-phosphate (GAP). The reaction mixture and 3-phosphate dehydrogenase as a function of the concentration of ELM (X—X) and 66 I.—M (A—A).

**Table VII**

| Coenzyme                     | Ratio of enzyme to coenzyme to iodosobenzoate in assay | Acetyl phosphate hydrolysed | Activity |
|------------------------------|--------------------------------------------------------|-----------------------------|----------|
|                              | mol/mol: mol/mol | µmol | %    |
| NAD                          | 1:200:0        | 0.1  | 2     |
| 3-Pyridinealdehyde-NAD       | 1:200:0        | 0.1  | 2     |
| NAD                          | 1:200:200      | 4.2  | 100   |
| 3-Pyridinealdehyde-NAD       | 1:200:200      | 2.9  | 69    |
| NAD                          | 1:50:200       | 4.1  | 98    |
| 3-Pyridinealdehyde-NAD       | 1:50:200       | 2.7  | 64    |

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 were measured on the control with no 3-pyridinealdehyde-NAD (●—●) and at 3-pyridinealdehyde-NAD concentrations of 33 µM (X—X) and 66 µM (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 polypeptide 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 alkylation 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). Preinactivation 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 [14C]iodoacetic acid showed that there is a slow time-dependent release of 3-pyridinealdehyde-NAD with binding of [14C]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 [14C]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 and iodosobenzoate to react with cysteine-149. If with 3-pyridinealdehyde-NAD instead of NAD may be caused by competition between the aldehyde group of 3-pyridinealdehyde-NAD and iodosobenzoate. A thiohemiacetal bond is formed between the apoenzyme is oxidized first, then the added NAD or 3-pyridinealdehyde-NAD and 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.

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 ultrafiltration 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 ultrafiltration 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 also 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.

Acknowledgments—The expert technical assistance of Miss Suzanne Brandon and Mrs. Blanche Meriwether is gratefully acknowledged. We are also grateful to Dr. Robert Post and Dr. Sidney Colowick for their critical review of the manuscript.

REFERENCES
1. Colowick, S. P., vanEys, J., and Park, J. H. (1966) in Comprehensive Biochemistry (Florkin, M., and Stotz, E. M., eds) Vol. 14, p. 55, Elsevier, Amsterdam
2. Cori, G. T., Slee, M. W., and Cori, C. F. (1948) J. Biol. Chem. 173, 605-618
3. Fox, J. B., and Dandliker, W. B. (1956) J. Biol. Chem. 211, 1005-1017
4. Taylor, J. F., Velick, S. F., Cori, C. T., Cori, C. F., and Slee, M. F. (1948) J. Biol. Chem. 173, 619-626
5. Park, J. H., Agnello, C. F., and Mathew, E. (1966) J. Biol. Chem. 241, 769-771
6. Velick, S. F., Hayes, J. E., Jr., and Harting, J. (1953) J. Biol. Chem. 203, 527-544
7. Stadtmann, E. R., and Lipmann, F. (1956) J. Biol. Chem. 156, 549-551
8. Yee, H. Y. (1965) Cita. Chem. 14, 898-903
9. Lipmann, F., and Tuttel, L. C. (1945) J. Biol. Chem. 159, 21-28
10. Allison, W. S., and Kaplan, N. O. (1964) Biochemistry 3, 1792-1800
11. Parker, D. J., and Allison, W. S. (1969) J. Biol. Chem. 244, 180-189
12. Racker, E., and Krimsky, I. (1958) Fed. Proc. 17, 1135-1144
13. Harris, I., Meriwether, D. P., and Park, J. H. (1963) Nature 198, 154-159
14. Mathew, E., Agnello, C. F., and Park, J. H. (1965) J. Biol. Chem. 230, PC 3232-PC 3235
15. Fenselau, A. (1970) J. Biol. Chem. 245, 1239-1246
16. Segal, H. L., and Boyer, P. D. (1953) J. Biol. Chem. 204, 395-391
17. Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1957) Arch. Biochem. Biophys. 60, 441-457
18. Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1956) J. Biol. Chem. 221, 883-841
19. Park, J. H., Meriwether, B. P., Clodfelder, P., and Cunningham, L. W. (1961) J. Biol. Chem. 236, 136-141
20. McDonald, G., Brown, B., Hollis, D., and Walter, C. (1972) Biochemistry 11, 1920-1930
21. Chandrasekhar, K., McPherson, A., Jr., Adams, M. J., Hackert, M. I., Lijja, A., Budney, M., Ford, G. C., and Rossmann, M. G. (1973) J. Mol. Biol. 76, 503-518
22. Webb, L. E., Hill, E. J., and Banaszak, L. J. (1973) Biochemistry 12, 5101-5109
23. Kaplan, N. O., and Ciotti, M. M. (1958) J. Biol. Chem. 231, 785-794
24. Dube, S. K., Koholt, O., and Pressman, D. (1965) J. Biol. Chem. 235, 613-617
25. Bonnichsen, K. K., and Brink, N. G. (1955) Methods Enzymol. 1, 498
26. Racker, E. (1955) Methods Enzymol. 1, 503
27. Varonco, S., Consiglio, E., and Covelli, L. (1970) Eur. J. Biochem. 12, 305-312
28. Rossmann, M. G., Adams, M. J., Buchner, M., Ford, G. C., Hackert, M. L., Lujan, P. J., Jr., McPherson, A., Jr., Schewitz, R. W., and Smiley, L. E. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 179-191
29. Hill, E. J., Tserngloou, L., Webb, L., and Banaszak, L. J. (1972) J. Mol. Biol. 72, 577-591
30. Branden, C. I., Ekland, H., Nordstrom, B., Bouw, T.,
Söderling, G., Zeppezauer, E., Ohlsson, I., and Åkeson, A. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2439-2442
31. Buehner, M., Ford, G. C., Moras, D., Olsen, K. W., and Rossmann, M. G. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 3052-3054
32. Rafter, G. W., and Colowick, S. P. (1957) J. Biol. Chem. 224, 373-379
33. Pihl, A., and Lange, R. (1962) J. Biol. Chem. 237, 1356-1362
34. Ehring, R., and Colowick, S. P. (1969) J. Biol. Chem. 244, 4559-4599
35. McPherson, A., Jr. (1970) J. Mol. Biol. 51, 39-46
36. Oguchi, M., Meriwether, B. P., and Park, J. H. (1973) J Biol Chem 248, 5562-5570
Covalent binding of 3-pyridinealdehyde nicotinamide adenine dinucleotide and substrate to glyceraldehyde 3-phosphate dehydrogenase.
E J Hill, T H Chou, M C Shih and J H Park

J. Biol. Chem. 1975, 250:1734-1740.

Access the most updated version of this article at http://www.jbc.org/content/250/5/1734

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/5/1734.full.html#ref-list-1