Interactions of 3-Aminopyridine Adenine Dinucleotide with Dehydrogenases*

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SUMMARY

NAD was converted chemically to 3-aminopyridine adenine dinucleotide through the Hofmann hypobromite reaction and the resulting dinucleotide was purified by means of ion exchange chromatography. Spectrophotometric and fluorometric properties of the analog were studied. The 3-aminopyridine adenine dinucleotide was shown to be a competitive inhibitor in reactions catalyzed by seven NAD(P)-dependent enzymes.

The 3-aminopyridine adenine dinucleotide was diazotized with the use of nitrous acid. The diazotized derivative was shown to be a site-specific inactivator of yeast alcohol dehydrogenase. Diazotized 3-amino-1-methylpyridinium chloride was prepared and shown to be less effective in the inactivation of this enzyme.

The preparation of site-labeling pyridine dinucleotide derivatives through the transglycosidation reactions (1) catalyzed by mammalian NADases is complicated by the fact that products formed should be inhibitory to the enzyme required for the synthesis. It was felt more advantageous to produce a pyridine dinucleotide derivative which in a second chemical reaction could be converted to a site-labeling reagent. For this reason, a study of the synthesis of 3-aminopyridine adenine dinucleotide (AAD) was undertaken. It should be noted that AAD† was synthesized previously (2) from NAD through the pyridine base exchange reaction catalyzed by pig brain NADase. Unfortunately, through this synthetic route, extremely low yields of AAD were obtained and further characterization of the compound, as well as studies of the binding of this compound to enzymes, was deferred until a sufficient quantity could be prepared.

Recent studies in this laboratory suggested that AAD could be prepared through direct chemical modification of NAD. The Holman procedure (3) for the colorimetric determination of nicotinamide involves a Hofmann degradation of the amide to 3-aminopyridine followed by diazotization and azo-coupling to form a dye. This procedure routinely has been applied to intact NAD, assuming that the alkaline conditions of the Hofmann degradation result in the hydrolysis of the nicotinamide riboside linkage yielding stoichiometric amounts of free nicotinamide for determination. However, it was noted that the dye formed from NAD in the Holman procedure had a different absorption maximum than that obtained with free nicotinamide, suggesting that a portion of the intact dinucleotide might be maintained through this procedure and that AAD could be an intermediate resulting from the initial Hofmann degradation step. Modification of the conditions used in this step resulted in a 68% yield of AAD, permitting the characterization and further study of this compound.

The reactions of importance to the present study are shown in Fig. 1.

EXPERIMENTAL PROCEDURE

Materials

Glucose 6-phosphate dehydrogenase, horse liver alcohol dehydrogenase, NAD, and 3-acetylpyridine adenine dinucleotide were obtained from Sigma Chemical Co.; yeast alcohol dehydrogenase and pig heart malate dehydrogenase were obtained from Worthington Biochemical Corp.; electrophoretically pure beef heart and beef muscle lactate dehydrogenase and fluorescent grade tris(hydroxymethyl)aminomethane were obtained from Boehringer Mannheim Corp.; 3-aminopyridine was obtained from Eastman Kodak Co. and sublimed before use; bull semen NADase was prepared by the method of Yuan and Anderson (4).

Some properties of 3-amino-1-methylpyridinium chloride have been reported (5, 6), but the method by which the com-
compound was obtained has not. Therefore, synthesis of the compound by the Menschutkin reaction is described. Sublimed 3-aminopyridine (0.94 g, 10 mmoles) and iodomethane (0.62 ml, 10 mmoles) were dissolved in 25 ml of acetone and the solution was refluxed for 2 days. After the solution was cooled to about 4°, the supernatant was decanted. The crystals were dissolved in H2O and eluted with H2O from a column containing 75 meq of Dowex AG 1-X2 chloride. Efficient having appreciable absorbance at 260 nm was evaporated to an oil under reduced pressure (30° bath) and dried under vacuum over CaCl₂. The resulting solid was recrystallized twice from absolute ethanol-dichloroethylether to give 1.05 g (72%) of white, hydroscopic crystals.

\[ \text{C₆H₇ClN₂} \]

Calculated: C 49.84, H 6.27, N 19.37
Found: C 50.05, H 6.81, N 19.59
M.p.: 205.5-207.5° (uncorrected)

Methods

Characterization of Compounds—Thin layer chromatography was performed by using Eastman Chromagram cellulose sheets containing fluorescent indicator; the solvent was 0.1 M acetic acid-95% ethanol (1:1 by volume). The spots were detected by ultraviolet light.

Hydrolysis by snake venom phosphodiesterase was carried out at 4° in 0.01 M Tris Cl buffer, pH 8.1, in the presence of 0.05 M magnesium acetate.

Spectrophotometric data were obtained using a Zeiss PMQ II spectrophotometer; fluorometric data were obtained using an Aminco Bowman epi-spectrophotometer.

Inhibition of Enzymes—With the exception of the NADase constants, which were determined titrimetrically with a Radiometer automatic titrimeter (7), all kinetic data were obtained at 25° with a Beckman ACTA III spectrophotometer with 10° suppression capabilities by observing the formation of reduced coenzyme at the appropriate wave length (340 nm for NADH and 365 nm for reduced 3-acetylpyridine adenine dinucleotide). The pH was measured at 25° with a Radiometer PHM 52 pH meter and type 202 C glass electrode. Coenzyme concentrations of 0.54, 0.69, 1.00, 1.54, and 3.85 × 10⁻⁴ M and inhibitor concentrations of 0.5 and 1.0 × 10⁻⁴ M were used to obtain data for the Lineweaver-Burk plots (8) while coenzyme concentrations of 0.54 and 3.85 × 10⁻⁴ M and inhibitor concentrations of 0.25, 0.50, 0.75, and 1.00 × 10⁻⁴ M were used to obtain data for the Dixon plots (9). The term “maximum AAD” is defined as the greatest concentration of AAD used in the analysis of a particular enzyme; it is listed below for each enzyme studied. The volume of reagents used for each kinetic run totaled 3 ml; reactions were initiated by addition of the enzyme. Least squares analyses performed by a computer provided the data actually used to determine the kinetic constants.

Specifically, the following conditions were used in the assays (all numbers refer to concentrations in the cuvette): yeast alcohol dehydrogenase: 6.6 pm enzyme, 100 nm ethanal, 500 μm AAD (maximum concentration), 50 nm sodium pyrophosphate, pH 8.0; horse liver alcohol dehydrogenase: 0.12 nm enzyme, 200 nm ethanal, 1.04 nm AAD (maximum concentration), 50 nm sodium glycinate, pH 9.6; beef heart lactate dehydrogenase: 1.9 nm enzyme, 40 nm lactate, 499 nm AAD (maximum concentration), 50 nm Tris-chloride, pH 8.1; beef muscle lactate dehydrogenase: 6.6 nm enzyme, 200 nm lactate, 302 μm AAD (maximum concentration), 50 nm Tris-chloride, pH 8.1; glucose 6-phosphate dehydrogenase: 0.0075 unit of enzyme, 4.0 mm glucose 6-phosphate, 1.03 mm AAD (maximum concentration), 50 mm sodium glycinate, pH 9.6; pig heart malate dehydrogenase: 15 nm enzyme, 10 mm malate, 1.03 mm AAD (maximum concentration), 50 mm sodium glycinate, pH 9.6; bull semen NADase (measured titrimetrically by the method of Yuan and Anderson (7)): 79 nm enzyme, 68.8 to 155 nm NAD, 58 nm NaCl, pH maintained at 7.5 by addition of NaOH.

RESULTS

Synthesis of AAD—The optimum conditions for the conversion of NAD to AAD by means of the Hofmann hypobromite reaction were ascertained only after detailed analysis of several phases of the reaction, most importantly the amount of Br₂ utilized and the reaction time required. Hypobromite was prepared in a hood by dissolving 0.077 ml (1.5 mmoles) of ice-cold Br₂ in 100 ml of vigorously stirred 0.5 M NaOH. The hypobromite was mixed with 400 ml of 2.5 mM NaCl to initiate the reaction. The progress of the reaction could be followed by observing the increase in fluorescence (excitation λ 323 nm, emission λ 420 nm) of aliquots of the reaction solution diluted with 0.1 M potassium phosphate buffer, pH 7.0. After 180 min at room temperature, the solution was adjusted to pH 7 with concentrated HCl and eluted by quasilinear gradient (mixer: 1 liter of H₂O; reservoir: 1 liter of 1.0 M ammonium formate) from a column (height/diameter = 10) containing 0.6 eq of Dowex AG 1-X8 formate. The eluent was monitored at 290 nm; the major peak occurred at an ammonium formate concentration of approximately 0.15 M. All fractions of this peak with an absorbance >2 were pooled and lyophilized. The resulting powder was stirred with 200 ml of absolute ethanol for 15 min and centrifuged at 650 × g at 4° for 20 min, after which the supernatant was discarded. The extraction was repeated and the remaining solid was dried under vacuum over CaCl₂ to give 0.498 g (68%) of white, hygroscopic powder. Thin layer chromatography of the compound yielded a single spot (Rf 0.54, blue fluorescence). Chromatography of the products of basic hydrolysis (0.5 M NaOH at 100° for 15 min) revealed two major products: a blue fluorescent spot with an Rf (0.81) identical with 3-aminopyridine, and a quenching spot with an Rf (0.60) identical with adenosine diphosphoribose; chromatography of the products of snake venom phosphodiesterase cleavage also gave two major products: a quenching spot with an Rf (0.66) identical with AMP and a blue fluorescent spot at an Rf (0.69) which is thought to be 3-aminopyridine mononucleotide. AAD was further characterized by quantitative determination of several of its functional groups, as shown in Table I.

| C₆H₇N₄O₁₄P₂ |
| Calculated: C 34.38, H 5.03, N 17.15 |
| Found: C 34.92, H 5.16, N 16.80 |

Thus, the structure of the compound actually isolated and employed in all of the investigations described in this report was consistent with 3-aminopyridine adenine dinucleotide diammonium mononucleotide monohydrate (molecular weight 733.52). Spectrophotometric and fluorometric characteristics of AAD are shown in Table II.

Since the spectral properties of pyridinium compounds are known to be influenced by the type of neutralizing anion (1, 13), a careful study of the effects of several anions on the fluorescence intensity of AAD was made. The results of this study are shown in Fig. 2.

To illustrate by analogy the feasibility of the reaction in a more easily characterized system, the Hofmann hypobromite
TABLE I
Quantitation of functional groups of AAD

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Mols/mole AAD</th>
<th>Theoretical ratio</th>
<th>Analytical method (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amines</td>
<td>0.85</td>
<td>1</td>
<td>Holman (3)</td>
</tr>
<tr>
<td>Adenines</td>
<td>0.97</td>
<td>1</td>
<td>Loring et al. (10)</td>
</tr>
<tr>
<td>Phosphates</td>
<td>2.09</td>
<td>2</td>
<td>Fiske and SubbaRow (11)</td>
</tr>
<tr>
<td>Riboses</td>
<td>2.04</td>
<td>2</td>
<td>Taylor et al. (12)</td>
</tr>
</tbody>
</table>

- This value was obtained after hydrolysis at 100° in 0.5 M NaOH for 15 min, using 3-aminopyridine as the standard. NAD fails to give a positive test, indicating the adenine amino group not to react under these conditions.
- A correction for 97.8% recovery of standard (adenine) was applied.
- Elut replaced the usual reducing agent in the modification used here.
- NAD was the standard.

TABLE II
Spectral properties of analogs at pH 7.0

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Absorption λmaxa</th>
<th>ε x 10⁻⁶b</th>
<th>Fluorescence λ emissionc</th>
<th>Quantum efficiencyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>282</td>
<td>7.8</td>
<td>401</td>
<td>0.10</td>
</tr>
<tr>
<td>Diazotized AM</td>
<td>282</td>
<td>3.56</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>AAD</td>
<td>282</td>
<td>11.1</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Diazotized AAD</td>
<td>261</td>
<td>19.6</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

- The solvent was 0.1 M potassium phosphate.
- The solvent was 1 mM sodium phosphate.
- The quantum yield was established by comparison with NADH under identical conditions and taking 0.019 as the quantum yield (14) for NADH.
- 3-Amino-1-methylpyridinium chloride (AM).

reaction was performed on 1 mmole of 1-methylnicotinamide chloride (15). The reaction was monitored fluorometrically (excitation λ 320 nm, emission λ 402 nm). After the reaction solution was neutralized, it was evaporated to dryness under reduced pressure (30° bath). Several extractions with absolute alcohol and evaporation of the supernatant gave a solid which was recrystallized from absolute ethanol-diethyl ether. The compound, isolated in 37% yield, was identical (thin layer chromatography, ultraviolet light, melting point) with authentic 3-amino-1-methylpyridinium chloride. Spectrophotometric and fluorometric properties of this derivative are also listed in Table II.

Inhibition of Enzymes by AAD—The inhibitory effect of AAD was studied using seven NAD(P)-dependent enzymes. In all cases studied, inhibition by AAD was observed to be competitive with respect to the pyridine nucleotide coenzyme employed and, in the case of NADase, competitive with respect to substrate. The coenzyme-competitive nature of the inhibition of horse liver alcohol dehydrogenase by AAD is demonstrated in Figs. 3 and 4. Inhibitor dissociation constants obtained with all of the enzymes studied are shown in Table III. The 3-amino 1-methylpyridinium chloride was studied under identical assay conditions, but no inhibition was detected with any of the enzymes tested.

FIG. 2. Effect of concentration of various anions upon the reciprocal of the relative fluorescence intensity of AAD. The data were obtained in 0.001 M sodium phosphate buffer, pH 7.0. Fluorescence measurements were made with excitation at 331 nm and emission at 430 nm. The data for the formate anion are not shown but are similar to that of phosphate.

FIG. 3. A typical Lineweaver-Burk plot showing competitive inhibition of horse liver alcohol dehydrogenase by AAD. Table III and the text provide the experimental details. The coenzyme used was 3-acetylpyridine adenine dinucleotide (APAD).

Diazotized AAD—The diazotization of AAD was carried out at 0°. To 0.05 ml of 60 mM AAD was added 0.25 ml of 1.0 M HCl and then 0.5 ml of 1.0 M NaN0₂. After 10 min, 0.5 ml of 2.0 M ammonium sulfate was added slowly with stirring in order to destroy the excess HNO₂. After an additional 10 min, 0.25 ml of 1.0 M NaOH and 2.0 ml of 0.10 M sodium pyrophosphate buffer, pH 7.0, were added.

An aliquot of the diazotized AAD solution was neutralized with NaOH and diluted to a concentration of 4.18 x 10⁻⁵ M with 0.1 M potassium phosphate buffer, pH 7.0. The absorption spectrum of this solution was compared to that for an equal concentration of AAD (Fig. 5). A comparison of these compounds at pH 8.0 gave spectra identical with those shown in Fig. 5. Under the same conditions, diazotized 3-amino-1-methylpyridinium chloride was prepared and compared spectrally at pH 7.0 to 3-amino-1-methylpyridinium chloride (Fig. 6). As in the case of the dinucleotide derivatives, the identical
Fig. 4. A typical Dixon plot showing inhibition of horse liver alcohol dehydrogenase by AAD. Table III and the text provide the experimental details. The coenzyme used was 3-acetylpyridine adenine dinucleotide (APAD).

Table III
Kinetic effects of competitive inhibitor AAD on seven enzymes

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Coenzyme*</th>
<th>$K_m$ (Lineweaver-Burk)</th>
<th>$K_I$ (Lineweaver-Burk)</th>
<th>$K_I$ (Dixon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YADH</td>
<td>NAD</td>
<td>228</td>
<td>702</td>
<td>571</td>
</tr>
<tr>
<td>HLADH</td>
<td>APAD</td>
<td>102</td>
<td>486</td>
<td>575</td>
</tr>
<tr>
<td>BHLDH</td>
<td>NAD</td>
<td>44.8</td>
<td>682</td>
<td>388</td>
</tr>
<tr>
<td>BMLDH</td>
<td>NAD</td>
<td>64.2</td>
<td>156</td>
<td>124</td>
</tr>
<tr>
<td>G-6-PDH</td>
<td>APADP</td>
<td>54.4</td>
<td>$\sim$ 4600</td>
<td></td>
</tr>
<tr>
<td>PHMDH</td>
<td>APAD</td>
<td>170</td>
<td>3250</td>
<td>1440</td>
</tr>
<tr>
<td>NADase</td>
<td></td>
<td>100</td>
<td>7.69</td>
<td></td>
</tr>
</tbody>
</table>

* Abbreviations for enzymes appearing in this table are: YADH, yeast alcohol dehydrogenase; HLADH, horse liver alcohol dehydrogenase; BHLDH, beef heart lactate dehydrogenase (M_4); BMLDH, beef muscle lactate dehydrogenase; PHMDH, pig heart malate dehydrogenase; NADase, bull semen NADase.

* Coenzymes APAD and APADP are 3-acetylpyridine adenine dinucleotide and its phosphate.

spectra were obtained at pH 8.0. The spectral data for diazo-
tized AAD appear to rule out the conversion of the adenine moiety to hypoxanthine under these conditions.

Inactivation of Yeast Alcohol Dehydrogenase by Diazotized AAD—To 4.0 ml of diazotized AAD solution (prepared as described in the previous section) was added 0.20 ml of 60 μM yeast alcohol dehydrogenase in 0.05 M potassium phosphate buffer, pH 7.5. The inactivation was monitored by assaying periodically under conditions shown under “Methods” for yeast alcohol dehydrogenase activity; the reaction was completed in about 60 min. A control solution, which lacked only AAD, showed no loss of activity during the same time interval. The sample and control were each dialyzed at 4°C against five 1-liter portions of 0.05 M potassium phosphate buffer, pH 7.5, over a 2-day period. The final enzyme concentration was determined from the absorbance of the control at 280 nm. The spectra of native and modified yeast alcohol dehydrogenase are compared in Fig. 7. By assuming the molar extinction coefficient at 262 nm to be the same for the residue formed by yeast alcohol dehydrogenase inactivation as for AAD itself, and correcting for the absorbance at 262 nm due to yeast alcohol dehydrogenase only, values of 3.5, 4.2, and 4.6 moles of AAD residues per mole of (tetrameric) yeast alcohol dehydrogenase were obtained in three independent experiments.

The inactivation of yeast alcohol dehydrogenase in the presence of excess diazotized AAD follows pseudo-first order kinetics and NAD protects the enzyme against inactivation (Fig. 8). At a concentration of $4.47 \times 10^{-4}$ M NAD, the rate of inactivation by diazotized AAD is decreased by 68.8%. Inactivation of yeast alcohol dehydrogenase by diazotized 3-amino-1-methylpyridinium chloride also follows pseudo-first order kinetics and in this case, 64.6% protection was observed with $4.47 \times 10^{-4}$ M NAD (Fig. 8). The rate of inactivation of yeast alcohol dehydrogenase was studied as a function of the concentration of the two diazonium derivatives. The effect of diazotized AAD concentration on the rate of inactivation is a sigmoidal relationship showing apparent saturation of the enzyme at high concentrations (Fig. 9). Diazotized 3-amino-1-methylpyridinium chloride is less effective as an inactivator of the enzyme and saturation kinetics were not observed within the limit of measurement of the fast rates obtained at high concentrations. The absence of linearity in the rate versus concentration relationships precluded any direct comparison of the relative effectiveness of the two diazonium derivatives. However, at equal concentrations ($1.2 \times 10^{-4}$ M), the rate of inactivation by diazotized AAD was approximately 11 times faster than that observed with diazotized 3 amino-1-methyl pyridinium chloride.
FIG. 6. Absorption spectra of $1.06 \times 10^{-4}$ M 3-amino-1-methylpyridinium chloride (---) and $1.06 \times 10^{-4}$ M diazotized 3-amino-1-methylpyridinium chloride (---) in 0.1 M potassium phosphate, pH 7.0.

The exponential effect on rate of inactivation at low concentrations of the diazonium derivatives was thought to be related to competing reactions of these compounds at pH 8.0. When diazotized AAD ($8.08 \times 10^{-5}$ M) was allowed to remain in 0.1 M potassium phosphate buffer, pH 8.0, for 20 min prior to the addition of yeast alcohol dehydrogenase, the rate of inactivation was reduced to 21% of the expected value (16). Likewise, a 20-min preincubation of diazotized 3-amino-1-methylpyridinium chloride at pH 8.0 reduced the rate of enzyme inactivation to 34% of the expected value. Incubation of these diazonium derivatives at pH 8.0 also decreases the ability of these compounds to azocouple with N-1-naphthylethylenediamine dihydrochloride. No spectral changes were observed during the 20-min incubation of the two diazonium derivatives.

DISCUSSION

Through the use of the Hofmann hypobromite reaction, NAD is converted rapidly to AAD. A noteworthy aspect of this conversion is its high efficiency, considering the complexity of dinucleotides and the harsh conditions of the reaction. The factors most critical to the realization of high yield are Br$_2$ concentration, reaction time, and (ethanol) extraction efficiency; therefore, deviation from the recommended conditions for these three factors should be avoided.

Once prepared, the dinucleotide is stable for several days in neutral solution and almost indefinitely in frozen solution or in dry, cool conditions as a solid. The very hygroscopic nature of AAD makes rapid handling essential.

FIG. 7. Absorption spectra of native (---) and modified (---) yeast alcohol dehydrogenase in 0.1 M potassium phosphate buffer, pH 7.0. The difference spectrum is also shown (.....).

FIG. 8. Pseudo-first order rates of inactivation of yeast alcohol dehydrogenase in 0.1 M potassium phosphate, pH 8.0. Line 1, $1 \times 10^{-4}$ M diazotized AAD; Line 2, $1 \times 10^{-4}$ M diazotized AAD plus $4.5 \times 10^{-4}$ M NAD; Line 3, $3 \times 10^{-4}$ M diazotized 3-amino-1-methylpyridinium chloride; Line 4, $3 \times 10^{-4}$ M diazotized 3-amino-1-methylpyridinium chloride plus $4.5 \times 10^{-4}$ M NAD.
A significant part of this investigation was devoted to an extensive characterization of the structure of AAD. The complexity of the structure precluded the use of some of the usual instrumental techniques; thus the assignment of structure depended to a great extent upon degradation of the dinucleotide and comparison of the fragments with standards. All analytical tests gave results consistent with a pure compound of the stated structure.

The electronic structure of AAD provides a sharp contrast to that of NAD; the former has a strongly electron-donating group attached to the pyridinium ring whereas the latter has a moderately electron-withdrawing group. Thus, resistance to cyanide anion addition and lack of enzyme-mediated reduction can be explained by the presence of the amine. Because of the meta relationship of the amino group of AAD to the positively charged ring nitrogen, no classic resonance structures may be drawn for the delocalization of the charge by shifts of the nonbonded electron pair from the amino- to the pyridinium nitrogen.

Nevertheless, the interaction between these two groups must be strong, because spectrophotometry reveals no pKₐ in the acid region (protonation of the amine) and a pKₐ of 12.20 for 3-amino-1-methylpyridinium chloride (deprotonation of the amine nitrogen).

The unusual electronic properties of this NAD analog prompted its use as an active site probe in NAD-dependent enzymes. A previous study indicated that the compound does not function as a coenzyme (17). Table III shows that AAD gives very significant inhibition with the seven enzymes tested while 3-amino-1-methylpyridinium chloride under the same conditions gives no detectable inhibition. The AAD displayed the least inhibition with glucose 6-phosphate dehydrogenase, the only NADP-requiring enzyme tested. The greatest inhibition was observed with NADase and was two orders of magnitude lower than most other NAD analogs tested previously with this enzyme (7).

One important factor which must be kept in mind when measuring the AAD fluorescence is the profound effect which nearly all anions have on the intensity of the fluorescence. Fig. 2 shows the effect of several anions and illustrates the inverse relationship of fluorescence intensity to anion concentration. Presumably the fluorescence is quenched through formation of a molecular complex, possibly of the charge-transfer type, a common phenomenon in compounds of this electronic structure (13).

A charge-transfer involving the pyrophosphate group of the dinucleotide may account for a portion of the 30 times less fluorescence of AAD compared with that of 3-amino-1-methylpyridinium chloride. Adenine probably contributes to the diminished fluorescence of AAD also, because the midpoint of the transition to 5 times greater fluorescent intensity on going from neutral to acidic conditions occurs at pH 3.9, an accepted pKₐ of adenine. This phenomenon is probably the result of unstacking of the rings due to charge repulsion and concomitant diminution of intermolecular interactions.

Quenching effects between solvent anions and AAD are reduced or eliminated on binding of the AAD to beef muscle lactate dehydrogenase or bull semen NADase, an indication that steric or electronic effects prevent accommodation of intermolecular complexes at the binding site. This suggests the possibility of observing very small values of fluorescence enhancement by conducting kinetic determinations in a highly quenching solvent. The technique has failed with yeast alcohol dehydrogenase, but investigations with other enzymes are continuing.

The most interesting derivative of AAD studied thus far is diazotized AAD. Besides providing an intermediate for the synthesis of other NAD analogs, it is itself a versatile reagent for enzyme studies. With yeast alcohol dehydrogenase, diazotized AAD acts as an inactivator. Evidence to support selective inactivation is as follows:

(a) Low concentrations of diazotized AAD rapidly inactivate the enzyme; (b) 4 molecules of diazotized AAD are bound per tetrameric molecule of yeast alcohol dehydrogenase; (c) NAD protects the enzyme against inactivation; (d) diazotized 3-amino-1-methylpyridinium chloride, a much smaller molecule possessing the same active group, inactivates yeast alcohol dehydrogenase at only 0.1 times the rate of diazotized AAD.

The sigmoidal relationship observed in the inactivation of yeast alcohol dehydrogenase by diazotized AAD is thought to be related to the conversion of the dinucleotide derivative to a form incapable of azo-coupling and enzyme inactivation. The formation of diazo-hydroxides or diazotates is characteristic of aryl diazonium derivatives containing electron withdrawing substituent groups (18). On the basis of studies of nitrobenzene diazotates (19), one would expect a time-dependent conversion of diazotized pyridinium derivatives to stable diazotizates. In the case of diazotized AAD, the resulting diazotate or diazo-hydroxide could be bound as a coenzyme-competitive inhibitor and thus protect the enzyme from inactivation. At low concentrations of diazotized AAD where the rate of enzyme inactivation is slower, the ratio of diazotized AAD to hydroxylated product would decrease allowing more of the enzyme to be converted to a protected complex, yielding a lower than expected rate of inactivation.

Diazotized 3-amino-1-methylpyridinium chloride is less effective as an inactivator of yeast alcohol dehydrogenase than is diazotized AAD. Since the present study suggests that an enzyme-binding process is involved in the inactivation of this enzyme by the diazonium derivatives, and since N⁺₃ alkylpyridinium derivatives are more poorly bound to yeast alcohol dehydrogenase than are pyridine dinucleotides (20-22), the lower effectiveness of diazotized 3-amino-1-methylpyridinium chloride can...
be attributed to a lower concentration of enzyme-diazonium derivative complex in a pre-equilibrium step of the inactivation process.

Studies are currently under way to identify the reaction responsible for alteration of the diazonium group of these compounds at pH 8.0 and to identify the amino acid residue(s) of yeast alcohol dehydrogenase modified during the inactivation process.

This investigation has shown that AAD is a versatile compound for the study of NAD-dependent enzymes and is worthy of further study.

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REFERENCES

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