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Preparation and Evaluation of Arylazide-Substituted Pyridine Adenine Dinucleotides for Photoaffinity Labeling Experiments

Wenqiu Liu, Michelle Gaines, Rong Bao, Jacquelynn Clifford and Yuzhou Li*

Department of Chemistry, Clarkson University, Potsdam, NY, USA

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ABSTRACT

Two pyridine-modified NAD$^+$ analogs, 3-(3-azido benzoyl) pyridine adenine dinucleotide 1 and *N*-(3-azido-5-carboxyl) phenyl nicotinamide adenine dinucleotide 2 have been prepared and evaluated for photoaffinity labeling experiments. The syntheses were accomplished *via* a mammalian NADase-catalyzed base exchange reaction. The new NAD$^+$ analogs retained the carbonyl or carboxamido functional group at the 3 position of the pyridine ring. The analog 1 is the first pyridine-modified azido derivative of NAD$^+$ that has shown coenzyme activity in a stereospecific hydride transfer reaction catalyzed by a dehydrogenase. Both NAD$^+$ analogs have shown potential for the study of active sites of NAD$^+$-utilizing enzymes.

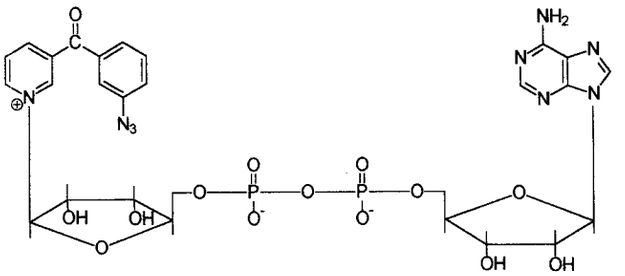
INTRODUCTION

Nicotinamide adenine dinucleotide (NAD$^+$)† is involved in numerous different types of enzyme-catalyzed reactions that play important roles in biological systems. In oxidation–reduction reactions, NAD-dependent enzymes catalyze a stereospecific transfer of hydride from a substrate to the nicotinamide ring of the dinucleotide to produce the 1,4-dihydronicotinamide moiety of the reduced coenzyme (NADH). Nicotinamide adenine dinucleotide is a substrate for various ADP-ribosyl transferases in ADP-ribosylation reactions (1), in which the ADP-ribosyl transferases transport the ADP-ribose moiety generated from the dinucleotide to acceptor molecules. Enzymes that utilize NAD$^+$ in these reactions usually have complex subunit structures and many of them are located in the unresolved structural complexes of mitochondria and chloroplasts. Therefore, the development of effective site-specific affinity labels for these NAD-dependent enzymes would allow the determination of the cellular locations of the enzymes and detection of the specific NAD binding sites of the proteins. Moreover, the chemistry of an NAD$^+$ analog can provide useful insight into the study of various NADP-dependent enzymes or proteins. In addition, an NAD$^+$ analog that has coenzyme activity could be used to produce the reduced form of the analog.

Photoaffinity labels represent a special category of labeling reagents (2–5). Upon irradiation, they give highly reactive intermediates that covalently bind to the receptor sites. The labeled enzyme then can be used in the study of the location of active sites. A number of photoactive NAD analogs have been prepared and used to locate the binding sites of different NAD-dependent enzymes. The analogs differ with respect to the position of the substitution of the photoactive group on the dinucleotide molecule. The alternations include modifications at either the adenine, ribose or pyridine portion of NAD$^+$. Examples of the analogs with a photoactive group on the adenine moiety of NAD$^+$ include 2-azidoadenosyl NAD$^+$ and 8-azidoadenosyl NAD$^+$ (6–9). These azidoadenine analogs generally have low quantum efficiencies of photoactivation due to the reversible azido-tetrazole equilibrium in aqueous solution. Analogs with a modification at the ribose portion of NAD$^+$ have been used to investigate several NAD$^+$-dependent enzymes (10–16) but they are subject to the hydrolytic cleavage of the ester linkage to the ribose (10,17). The enzymatic assay for such analogs has to be conducted at a certain pH to avoid the hydrolytic cleavage of the ester linkage regardless of the optimum pH for the specific enzymatic reaction. The modification at the pyridine portion of NAD$^+$ mainly involves the substitution of a 3-carboxamide group of the nicotinamide moiety of the dinucleotide with a diazocarbonyl (18), an azido (19) and a diazirine group (20). Although they are competitive inhibitors of natural NAD$^+$ and have shown various degrees of photoaffinity labeling ability, none of these analogs retains any coenzyme activity. It has been shown that the carboxamido group at the 3-position of the pyridine is essential to the proper alignment in the active site and the coenzyme activity of NAD$^+$. Substitutions other than the carboxamide group may cause steric difficulties in the orientation of analogs in the enzyme (21). Analogs structurally altered in the pyridine moiety of the dinucleotide are more likely to influence the function of analogs in the stereospecific hydride transfer reaction catalyzed by dehydrogenase. Therefore, it is of particular interest to study the pyridine-modified analogs. In addition, the preparation of pyridine-modified NAD$^+$ is extremely simple compared to a lengthy synthesis for an adenine- or ribose-modified analog. The

*To whom correspondence should be addressed at: Department of Chemistry, Clarkson University, Box 5810, Potsdam, NY 13699, USA. Fax: 315-268-6610;
e-mail: yuzhuoli@cadvax.clarkson.edu.

†*Abbreviations:* GC, gas chromatography; LADH, equine liver alcohol dehydrogenase; mp, melting point; MS, mass spectroscopy; NAD$^+$, nicotinamide adenine dinucleotide; NADH, reduced NAD$^+$; TLC, thin-layer chromatography.



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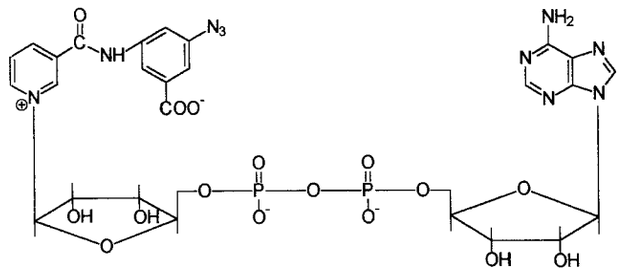

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Figure 1. Structures of 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1** and *N*-(3-azido-5-carboxyl)phenyl nicotinamide adenine dinucleotide **2**.

synthesis can be easily accomplished *via* a mammalian NADase-catalyzed base exchange reaction between a natural NAD^+ and a modified pyridine molecule (22–29).

The main objective of this study is to prepare and evaluate pyridine-modified NAD^+ that retains the carbonyl or carboxamido functional group at the 3-position of the pyridine ring of the dinucleotide. In this paper, we will first describe the preparation and characterization of two pyridine-modified analogs, 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1** and *N*-(3-azido-5-carboxyl)phenyl nicotinamide adenine dinucleotide **2** (Fig. 1). The analogs will be evaluated for their photoaffinity labeling ability with an equine liver alcohol dehydrogenase (LADH) through competitive inhibition and photoinhibition experiments. The results reported here indicate that both NAD^+ analogs are useful for the study of active sites of NAD^+-utilizing enzymes. Analog **1** is the first pyridine-modified azido derivative of NAD^+ showing coenzyme activity in a stereospecific hydride transfer reaction catalyzed by a dehydrogenase.

MATERIALS AND METHODS

LADH (EC 1.1.1.1) and porcine brain NADase (NAD^+ glycohydrolase, EC 3.2.2.5) were purchased from Sigma with the specific activities of 2 units/mg and 0.007 unit/mg, respectively. β-Nicotinamide adenine dinucleotide (NAD^+), NADH and adenosine 5′-diphospho ribose were obtained from Sigma. 3,5-Diamino benzoic acid and 3-benzoylpyridine were purchased from Lancaster and Aldrich, respectively. All other reagents and solvents were of the highest available purities and were used without further purification.

Synthesis of 3-(3-azidobenzoyl)pyridine. 3-Benzoylpyridine (6.3 g, 34 mmol) was added gradually to a mixture of fuming nitric acid (10 mL) and sulfuric acid (97%, 14 mL) at 0°C. The mixture was stirred for 4 h at room temperature. After neutralization, the mixture was extracted with chloroform (2 × 40 mL). The chloroform phases were combined and dried. Evaporation of chloroform under reduced pressure yielded 6 g (78%) of 3-(3-nitrobenzoyl)pyridine. Gas chromatography/mass spectroscopy (GC/MS) m/z = 228 (M^+); IR 1656 (C = 0) cm^{-1}.

Palladium on activated charcoal (5%, 0.2 g) was added slowly to a solution of 3-(3-nitrobenzoyl) pyridine (1 g, 4.4 mmol) in ethanol (95%, 40 mL). A flow of hydrogen was passed through the suspension for 20 h. The mixture was filtered and concentrated under reduced pressure to obtain a brown residue. The residue was then chromatographed on a silica gel column with petroleum ether/ethyl acetate (2:1) as an eluent to obtain 0.35g (40%) of 3-(3-aminobenzoyl)pyridine. Melting point (mp) 94–96°C; lit. mp 93–95°C (30); GC/MS m/z = 198 (M^+); IR 1661 (C=O) cm^{-1}; ^1H NMR(250 MHz, $CDCl_3$) δ 8.98 (s, py, 1H), δ 8.79 (d, py, 1H), δ 8.09 (d, py, 1H), δ 8.00 (s, ph, 2H), δ 7.42 (t, py, 1H), δ 7.27 (s, ph, 1H), δ 6.91 (t, ph, 1H), δ 3.69 (s, N-H, 2H).

To 15 mL of 0.08 *M* aqueous solution of 3-(3-aminobenzoyl) pyridine, sulfuric acid (97%, 2 mL) was added slowly at 0°C. The 12 mL of sodium nitrite solution (1.6 *M*) was added dropwise to the mixture. After removal of the excess nitrous acid with urea, 6 mL of sodium azide solution (3.3 *M*) was added to the mixture at 0°C over a period of 15 min. After the evolution of nitrogen, the mixture was stirred at 0°C for another 20 min and neutralized with a sodium hydroxide solution (50%, wt/wt). The mixture was extracted with chloroform (2 × 10 mL). The organic phases were combined, dried and concentrated under reduced pressure to yield 0.2 g of 3-(3-azidobenzoyl)pyridine. Melting point 95–97°C. IR 2089 (N_3) cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$) δ 8.99 (s, py, 1H), δ 8.84 (d, py, 1H), δ 8.13 (d, py, 1H), δ 7.49 (m, py and ph, 4H), δ 7.30 (d, ph, 1H).

Preparation of 3-(3-azidobenzoyl)pyridine adenine dinucleotide 1. 3-(3-Azidobenzoyl)pyridine (0.030 g, 0.21 mmol) was added to 3 mL of phosphate buffer (0.05 *M*, pH 7.5). To this mixture were added NAD^+ (0.028 g, 0.042 mmol) and porcine brain NADase (0.050 g). The entire mixture was incubated at 35°C for 6 h. The enzyme was removed from the reaction mixture by centrifugation. The reaction mixture was analyzed by HPLC. The suspected product was either collected from HPLC eluent line directly or separated on thin-layer chromatography (TLC) with methanol/water (85:15) as an eluent.

To conduct an NADase-catalyzed hydrolysis reaction of the synthesized analog, a mixture of porcine brain NADase (12.1 mg) and the suspect analog **1** solution (6.4 × 10^{-4} *M*, 1.3 mL) was incubated at 35°C for 3.5 h. The NADase was removed by centrifugation. The reaction mixture was analyzed by HPLC.

Synthesis of 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoic acid. Methyl 3,5-diamino benzoate (0.5 g, 3.01 mmol), nicotinoyl chloride hydrochloride (0.3 g, 1.68 mmol), triethylamine (1.22 g, 12 mmol), 4-dimethylaminopyridine (50 mg, 0.41 mmol) and methylene chloride (35 mL) were mixed together. The mixture was stirred at room temperature for 22 h and washed with water (2 × 35 mL). After removal of methylene chloride under reduced pressure, 0.4 g of a yellow solid was obtained. The crude product was purified by chromatography on silica gel first with ethyl acetate/petroleum ether (6:4) followed by ethyl acetate. The product was eluted off the silica gel with ethyl acetate. Evaporation of ethyl acetate under reduced pressure yielded 0.27 g (67.5%) of methyl 3-amino-5-[(3-pyridnylcarbonyl)amino]benzoate. GC/MS m/z=271 (M^+).

Methyl 3-amino-5-[(3-pyridinylcarbonyl)amino]benzoate (0.10 g, 0.55 mmol) was slowly added to 3 mL of sulfuric acid solution (32%, vol/vol) at 0°C. Then 2.5 mL of sodium nitrite solution (0.28 *M*, 0.7 mmol) was added dropwise to the mixture over a period of 5 min. The reaction mixture was then stirred at 0°C for 15 min. After decomposition of excess nitrous acid with urea, 2.5 mL of sodium azide solution (0.36 *M*, 0.9 mmol) was added dropwise into the mixture in 5 min. The reaction mixture was stirred at 0°C for 60 min and then filtered to yield 0.10 g of methyl 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoate. IR 2121 (N_3) cm^{-1}, GC/MS m/z = 297 (M^+); ^1H NMR (250 MHz, CD_3OD) δ 9.42 (s, py, 1H), δ

9.17 (d, py, 1H), δ 9.05 (s, py, 1H), δ 8.26 (d, py, 1H), δ 8.18 (s, ph, 1H), δ 7.88 (s, ph, 1H), δ 7.50 (s, ph, 1H), δ 3.93 (s, OCH_3 3H).

Methyl 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoate (84 mg, 0.27 mmol) was dissolved in 6 mL of methanol. Then 6 mL of 0.18 *M* NaOH solution was added to the solution. The entire mixture was stirred at room temperature for 21 h. As indicated by HPLC analysis, the hydrolysis reaction was completed. After methanol was removed under reduced pressure, a phosphoric acid solution (0.27 *M*) was added to the reaction mixture to adjust the pH into 4.8 and 65 mg of 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoic acid precipitated from the reaction mixture at this pH. Yield: 90%. GC/MS m/z = 283 (M^+); IR 2114 (N_3) cm^{-1}.

Preparation of N-*(3-azido-5-carboxyl)phenyl nicotinamide adenine dinucleotide* **2**. 3-Azido-5-[(3-pyridinylcarbonyl)amino]benzoic acid (35 mg, 0.12 mmol) was dissolved in 2.5 mL of phosphate buffer solution (0.05 *M*, pH 7.5). Then β-NAD^+ (13.9 mg, 0.084 mmol) and porcine brain NADase (50 mg) were added to the solution. The entire mixture was incubated at 35°C for 9 h and NADase was removed from the reaction mixture by centrifugation. The reaction mixture was analyzed by HPLC. The product was separated using a similar procedure for NAD^+ analog **1**. A similar enzymatic hydrolysis reaction was also performed on the new analog.

Assay of LADH activity. The LADH activity was assayed at room temperature by measuring the rate of NADH production at 340 nm. The standard assay mixture contained 56.4 μ*M* NAD^+, 62.3 m*M* Trizman buffer (pH 8.8), 0.21 *M* ethanol and 2.8 μg/mL LADH. The reactions were initiated by the addition of ethanol solution. The other assay conditions were indicated in the related figures. Specific activity is expressed as micromoles of NADH formed per minute per milligram of protein.

Photoinactivation of LADH. Photoinactivation of LADH by analog **1** or **2** was conducted at 0°C and pH 7.5. In a final volume of 340 μL, a phosphate buffer solution containing 23.5 μg/mL LADH and 0.94 m*M* analog **1** or 0.15 m*M* analog **2** was incubated 5 min at room temperature in a spot well plate, then cooled to 0°C on ice and irradiated with a shortwave (254 nm) UV lamp (model F8T5, So. N. E. Ultraviolet Co.) held at a distance of 30 cm from the sample. After photoirradiation, a 100 μL aliquot of the mixture was removed and assayed for activity.

Protection of LADH from photoinactivation by analog **1** *or* **2** *with the addition of* NAD^+ *prior to the photolysis.* The protection experiment was carried out by irradiation of the reaction mixture in the presence of NAD^+ (30–450 μ*M*). The reaction mixture contained LADH (0.048 mg/mL), 25 μ*M* analog **1** or 21 μ*M* analog **2**, and 12 m*M* phosphate buffer, pH 7.5. The reaction was initiated by adding LADH to the solution of analog **1** or **2**, and NAD^+, and the mixture was immediately exposed to the irradiation from a UV lamp (254 nm) held at a distance 30 cm at 0°C. After 5 min, a 100 μL aliquot was withdrawn and assayed for enzyme activity.

RESULTS

3-(3-Azidobenzoyl)pyridine and 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoic acid were synthesized from 3-benzoylpyridine and methyl 3,5-diamino benzoate, respectively as described in the Materials and Methods. The azido-modified pyridines were further used to prepare 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1** and *N*-(3-azido-5-carboxyl)phenyl nicotinamide adenine dinucleotide **2** by reacting with NAD^+ through an NADase-catalyzed base exchange reaction. The affinity and the photolabeling ability of the synthesized NAD^+ analogs were then studied.

Preparation of 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1**. The NAD^+ analog **1** was prepared by replacing the nicotinamide moiety of NAD^+ with 3-(3-azidobenzoyl)pyridine through a porcine brain NADase-catalyzed base exchange reaction. Chromatography of the reaction mixture by HPLC showed the presence of a new component in addition to NAD^+, nicotinamide, ADP-ribose and 3-(3-azidobenzoyl) pyridine. The new component was assigned to 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1**. The IR spectrum of the assigned analog shows a strong absorption at 2117 cm^{-1}, which is the characteristic absorption band of an azido group. Formation of ADP-ribose in the base exchange reaction was due to a breakage of pyridinium ribotide bond of either NAD^+ or the newly formed NAD^+ analog by the glycohydrolase activity of NADase.

In order to confirm the structure of the synthesized NAD^+ analog **1**, an NADase-catalyzed hydrolysis reaction was conducted. The mixture of NAD^+ analog **1** and NADase was incubated at 35°C and pH 7.5 for 3.5 h. Chromatography of the reaction mixture by HPLC shows only presence of 3-(3-azidobenzoyl)pyridine and ADP-ribose. The assigned NAD^+ analog **1** disappeared completely after 3.5 h of incubation. 3-(3-Azidobenzoyl)pyridine and ADP-ribose were formed by the breakage of pyridinium ribotide bond of NAD^+ analog **1** through the hydrolysis by the glycohydrolase activity of NADase. The ADP-ribosyl transferases are known to possess NAD glycohydrolase activity (31). The successful performance of the NADase-catalyzed hydrolysis reaction strongly suggests that the synthesized analog has a structure of 3-(3-azidobenzoyl) pyridine adenine dinucleotide.

Preparation of N-*(3-azido-5-carboxyl)phenyl nicotinamide adenine dinucleotide* **2**. The NAD^+ analog **2** was prepared by reaction of 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoic acid with NAD^+ under the catalysis of porcine brain NADase. In this enzymatic base exchange reaction, 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoic acid replaces the nicotinamide moiety of NAD^+ to form the NAD^+ analog. Chromatograph of the reaction mixture indicated formation of a new compound in addition to nicotinamide, ADP-ribose, NAD^+ and 3-azido-5-[(3-pyridinylcarbonyl)amino] benzoic acid. The new compound has a characteristic IR absorption of azido group and is assigned as *N*-(3-azido-5-carboxyl)phenyl nicotinamide adenine dinucleotide **2**. An NADase-catalyzed hydrolysis reaction of the assigned analog was also performed. After 9 h, the assigned analog **2** was decomposed completely to form ADP-ribose and 3-azido-5-[(3-pyridinylcarbonyl)amino]benzoic acid due to the breakage of pyridinium ribotide bond of the dinucleotide through the glycohydrolase activity of NADase.

Chemical and spectroscopic properties of NAD analogs. Each NAD^+ analog can be separated from the exchange reaction mixture by either the HPLC or TLC method. The solution was then concentrated under reduced pressure before use. The azido groups of the analogs were stable to normal fluorescent room light during the work-up. According to the HPLC analyses, both analogs are stable for at least several months when they are stored in the freezer. The UV absorption spectra of 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1** shows maxima at 232 nm, 260 and 282 nm and a shoulder at 318 nm. *N*-(3-azido-5-carboxyl)phenyl nicotinamide adenine dinucleotide **2** has UV absorption maxima at 208 nm, 230 nm, 258 nm and a shoulder at 318 nm. The concentration of the analogs was estimated from their UV absorption spectra by assuming that the molar absorptivities at 260 nm are the same for all NAD^+ analogs.

With LADH as the enzyme, analog **1** functions as a coenzyme in the enzymatic oxidation reaction of ethanol. The enzymatic assay of analog **1** was monitored with UV spectroscopy. Figure 2 shows growth of the UV absorption at 376 nm during the course of the enzymatic assay. The new

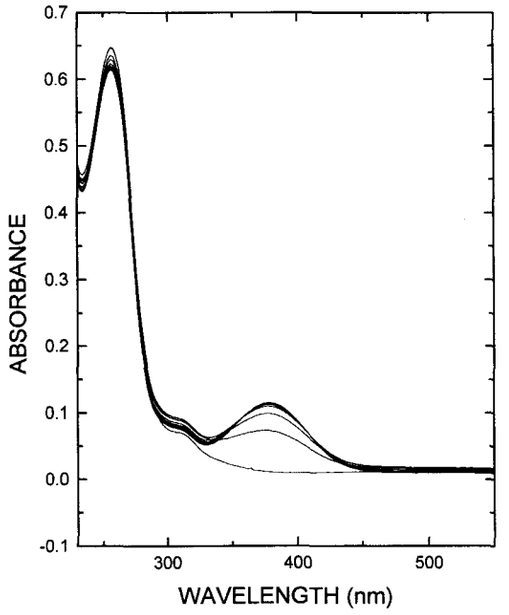

Figure 2. Absorption spectra of the reduced form of NAD⁺ analog **1** at 1 h intervals. The NAD⁺ analog **1** was converted to its reduced form by incubating with equine LADH with ethanol as substrate at room temperature for 12 h. The substrate concentration used was 0.2 *M* ethanol and 37 μ*M* NAD⁺ analog **1** in 65 m*M* Trizman buffer (pH 8.8).

absorption was assigned to the reduced form of analog **1**. The absorption maximum is *ca* 40 nm red shifted compared to natural NADH. It may be the result of higher conjugation formed between the pyridine ring and the added phenyl ring. A similar enzymatic assay, however, has shown that NAD⁺ analog **2** cannot be reduced enzymatically by LADH.

Inhibition of LADH. When NAD⁺ analog **1** or **2** was added to a normal enzymatic oxidation mixture containing natural NAD⁺, LADH and ethanol, the rate of production of NADH was decreased. It demonstrates that both analogs **1** and **2** compete with natural NAD⁺ for the active site. Kinetic studies indicate that both NAD⁺ analogs are linear competitive inhibitors of LADH with inhibitor dissociation constants, K_i, of 36.3 μ*M* and 49.4 μ*M*, respectively. In comparison with the dissociation constant of natural NAD⁺, both analogs have a similar affinity as natural coenzyme to the active site of the dehydrogenase.

Photoinactivation of LADH by analog 1. Irradiation of a mixture of NAD⁺ analog **1** and dehydrogenase with 254 nm light for 5 min resulted in a 69% decrease of the enzyme activity (Table 1). In a control experiment, an irradiation of the dehydrogenase alone under the same irradiation condition did not cause such a decrease of the enzyme activity. In addition, the preirradiated NAD⁺ analog **1** did not show a significant inhibition effect on the dehydrogenase.

Addition of NAD⁺ to the mixture of the dehydrogenase and NAD analog **1** prior to the irradiation resulted in a significant decrease in photoinactivation of the dehydrogenase. The protection from photoinactivation afforded by NAD⁺ increased when the concentration of NAD⁺ was increased. The effect finally saturated at about 20 μ*M*, a concentration in the vicinity of the dissociation constant of NAD⁺, K_M.

Table 1. Photoinactivation of equine LADH by NAD⁺ analogs

Irradiation conditions	Enzyme activity* (%)
LADH: in the dark	100
LADH: irradiated	96
LADH and analog **1**: in the dark	76
LADH and analog **1**†: irradiated	31
LADH and preirradiated analog **1**‡: in the dark	68
LADH and preirradiated analog **1**: irradiated	84
LADH and analog **2**: in the dark	94
LADH and analog **2**§: irradiated	30
LADH and preirradiated analog **2**‡: in the dark	72
LADH and preirradiated analog **2**: irradiated	86

*Aliquots (300 μL for analog **1** and 159 μL for analog **2**) were taken and assayed with the standard assay condition as described in the Materials and Methods. The relative enzyme activity is calculated with setting specific enzyme activity of the standard enzymatic assay as 100%.

†A solution (600 μL) containing 13.3 μg/mL equine LADH, 0.54 m*M* analog **1** and 33 m*M* Trizman buffer (pH 8.8) was placed in a cavity of a porcelain spotwell plate, incubated for 5 min at room temperature and irradiated at 0°C for 5 min with a short-wave (254 nm) UV lamp held at a distance of 30 cm from the sample.

‡A phosphate buffer solution of NAD⁺ analog **1** (0.70 m*M*) or analog **2** (0.87 m*M*) was preirradiated with 254 nm UV light for 15 min until the HPLC spectrum indicated that all the analog was converted to its photoproduct. A portion of this preirradiated solution was immediately used for the photoinactivation test.

§A solution (318 μL) containing 25.2 μg/mL LADH, 0.16 m*M* analog **2** and 68.6 m*M* Trizman buffer (pH 8.8) was irradiated with the same conditions used for analog **1**.

In order to assure that the photoinactivation was truly due to photoaffinity labeling of the NAD⁺ analog to the enzyme, a series of experiments was performed. The Lineweaver–Burk plot, shown in Fig. 3, displays three sets of experiments. In set I, the concentration of NAD⁺ in each assay was varied, and no analog was added. In set II, the same variable substrate concentrations were used as in set I, and a constant amount of analog was added to each assay. In set III, the same variable substrate concentrations were used as in sets I and II, the enzyme was first irradiated with 254 nm light for 5 min in the presence of the same amount of analog as in set II. The experiment set I and II demonstrated that NAD⁺ analog **1** competitively inhibited the enzyme activity when it was incubated with the enzyme in the dark. The addition of higher concentrations of the natural substrate resulted in a reversal of such inhibition by replacing the NAD⁺ analogs from the active site of the enzyme. The catalytic ability of the enzyme was not altered and the same V_{max} value was kept in experiment set II. However, this characteristic competitive inhibition disappeared after irradiation of the dehydrogenase with the analog. The addition of higher concentrations of the natural substrate NAD⁺ was incapable of reversing the inhibitory effect because NAD⁺ cannot replace the NAD⁺ analogs from the active sites of the enzyme due to the formation of the covalent bonding. The V_{max} value of experiment set III decreased 48% compared with set I and II, which indicates that a significant portion of the enzymatic catalytic ability of the dehydrogenase was permanently damaged.

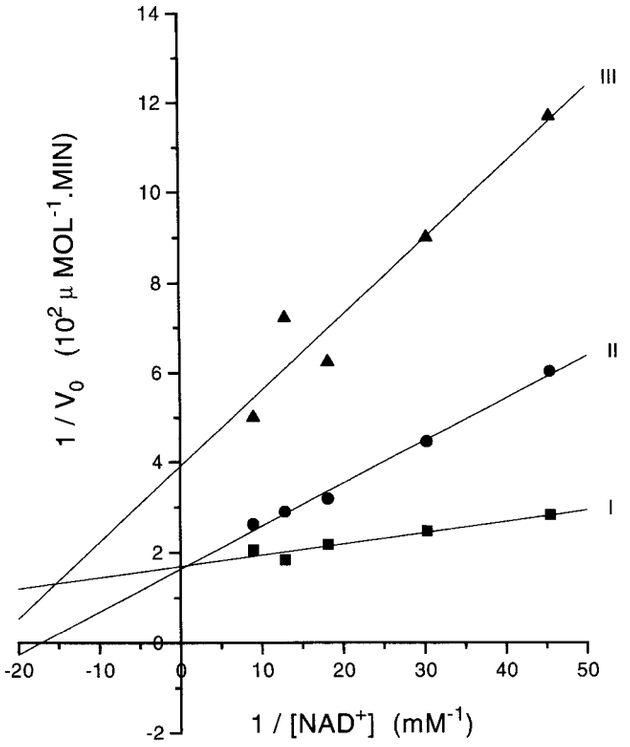

Figure 3. A double-reciprocal kinetic plot of LADH subject to reversible competitive inhibition and photoinactivation by NAD^+ analog **1**. The assay procedure was that described in the Materials and Methods utilizing different concentrations of NAD^+ with 2.7 μg/mL of LADH in the presence of NAD^+ analog **1** with a concentration of 0 m*M* (I), 0.11 m*M* (II) and 0.11 m*M* (III). The photoinactivation (III) was performed with a phosphate buffer solution (340 μL) containing 23.5 μg/mL LADH and 0.94 m*M* NAD analog **1**, which was exposed to the irradiation as described in the session of photoinactivation of LADH in the Materials and Methods.

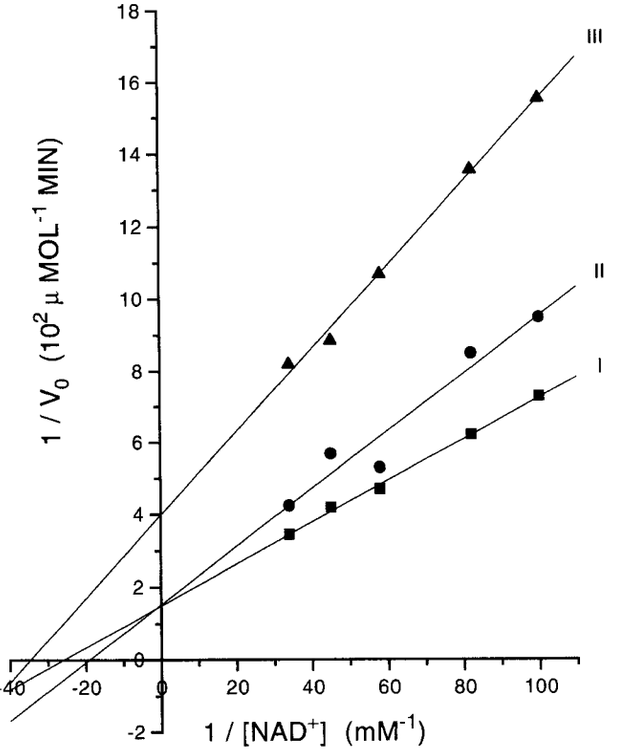

Figure 4. A double-reciprocal kinetic plot of LADH subject to reversible competitive inhibition and photoinactivation by NAD^+ analog **2**. The assay procedure was that described in the Materials and Methods utilizing different concentrations of NAD^+ with 2.7 μg/mL of LADH in the presence of NAD^+ analog **2** with a concentration of 0 m*M* (I), 17.3 M (II) and 17.3 M (III). The photoinactivation (III) was performed with a phosphate buffer solution (340 μL) containing 23.5 μg/mL LADH and 0.15 m*M* NAD analog **2**, which was exposed to the irradiation as described in the session of photoinactivation of LADH in the Materials and Methods.

Photoinactivation of LADH by analog 2. The same experiments that had been done for NAD^+ analog **1** were conducted for analog **2**. Irradiation of the mixture of the dehydrogenase and NAD^+ analog **2** with 254 nm light for 5 min caused a 70% loss of the enzymatic activity (Table 1). The enzyme activity was not substantially decreased by either irradiation of dehydrogenase alone, or irradiation of the mixture of dehydrogenase and the preirradiated NAD^+ analog **2**.

To confirm that a site-specific photoinactivation actually took place, protection studies were done by the addition of NAD^+ to the mixture of the dehydrogenase and NAD^+ analog **2** prior to irradiation. Addition of the natural substrate resulted in a decreased photoinactivation of the dehydrogenase. Such protection was enforced by the increase of the concentration of NAD^+ and finally reached saturation at the concentration in the vicinity of the substrate K_m.

A set of kinetic experiments (Fig. 3) similar to those for dinucleotide **1** demonstrated that analog **2** is a competitive inhibitor of NAD^+ for LADH while incubated with the enzyme in the dark. When a mixture of the dehydrogenase and NAD^+ analog **2** was exposed to irradiation, the characteristics of competitive inhibition of analog **2** disappeared. Formation of covalent linkage with the enzyme made analog **2** a noncompetitive inhibitor after irradiation. Addition of higher concentrations of NAD^+ did not recover the enzyme activity as it did without irradiation.

DISCUSSION

Synthesis of NAd^+ analogs

The NAD^+ analogs varying in the nature of the substituent group or the substitution position on the pyridine ring can be easily prepared by the mammalian NADase-catalyzed base exchange reaction. The enzymatic base exchange reaction has been used here to prepare 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1** and *N*-(3-carboxyl-5-azido)phenyl nicotinamide adenine dinucleotide **2**. The base exchange reaction provides a good yield of NAD^+ analog under mild reaction conditions. The solubility of the modified pyridine in aqueous solution is crucial to achieve a successful base exchange reaction. The solubility of a modified pyridine can be enhanced by introducing a hydrophilic group to the compound, adding an organic solvent, such as dimethylsulfoxide, to the buffer solution of the reaction, or varying pH of the reaction mixture. The modified pyridine for analog **1** has a reasonable solubility in the buffer solution and gives a high yield for the enzymatic exchange reaction.

The initial attempt to prepare analog **2** with *N*-3-azidophenyl-3-pyridine carboxamide failed. The poor solubility of such a compound in aqueous solution resulted in a low yield of the base exchange reaction. The yield was not improved significantly with either addition of organic solvent or variation of pH of the reaction mixture. The preparation of analog **2** was accomplished by introducing a carboxylic group to the phenyl ring of the pyridine compound to enhance its solubility in water.

The structures of 3-(3-azidobenzoyl)pyridine adenine dinucleotide **1** and *N*-(3-carboxyl-5-azido)phenyl nicotinamide adenine dinucleotide **2** are generally slightly less polar than NAD^+, ADP-ribose and nicotinamide but more polar than their parent compounds. Therefore, they can be easily separated from the exchange reaction mixture by using an HPLC or a TLC method. Due to the unique possession of an azido functional group, the isolated dinucleotides can be characterized by their IR absorption at *ca* 2100 cm^{-1}. Incubation of the NAD^+ analogs with porcine brain NADase results in formation of ADP-ribose and their corresponding parent pyridine compounds as products of breakage of pyridinium ribotide bond of analogs. Because the enzymatic hydrolysis is highly specific and efficient, it provides a simple and reliable method for structural confirmation of the prepared NAD^+ analogs.

Coenzyme activity of the NAD^+ analogs

LADH is composed of two subunits that utilize NAD(H) as a substrate. LADH is one of the most studied dehydrogenases, whose crystal structure is known and whose electron density map has been determined both in the presence and absence of NAD^+ (32). Among the various dehydrogenases that were used to investigate coenzyme-functioning of NAD^+ analogs, LADH appears to be a more versatile enzyme system for evaluation of the property of NAD^+ analogs (21).

It is of particular interest to note that 3-(3-azido benzoyl)pyridine adenine dinucleotide **1** functions as a coenzyme for LADH with ethanol as a substrate. Although several NAD^+ analogs prepared as photoaffinity labels have been shown to function as coenzymes (33), none of those analogs contained any modification on the pyridine ring of the dinucleotide. Structural alterations in the functional pyridine moiety of the dinucleotide are more likely to influence the binding, redox and kinetic properties of the analogs. Any modification on the pyridine ring could change the proper orientation and the redox potential of the ring, and thus its capability to function as a hydrogen acceptor in enzymatic reactions is affected. Although the pyridine moiety of the analog **1** is modified with a bulky azidophenyl group, it does retain the coenzyme activity. This can probably be attributed to retention of the most important carbonyl functional group at the C-3 position of the pyridine moiety, which could form the hydrogen bond with the enzyme to support effective binding and participation in the catalytic process (21). In comparison, 3-azido or 3-diazirino pyridine adenine dinucleotides have been found inactive as a coenzyme for dehydrogenases (18–20). The inability of analog **2** to function as a coenzyme is probably due to the presence of a carboxylic group, which may cause an improper positioning of the pyridine moiety of the dinucleotide.

Both NAD^+ analogs **1** and **2** are potent competitive inhibitors of LADH. The inhibition ability of both NAD^+ analogs **1** and **2** indicates their affinity toward the dehydrogenase despite the presence of bulky arylazide groups. To a certain extent, it shows a tolerance of the dehydrogenase toward the spatial extension of the carbonyl group of the pyridine moiety and the importance of the presence of the carbonyl group in the functional pyridine region.

Photoinduced inactivation of LADH

In addition to acting as competitive inhibitors of LADH, analog **1** and **2** could also cause permanent inactivation of the dehydrogenase upon irradiation (Table 1). Irradiation of the mixture of dehydrogenase and NAD^+ analog **1** or **2** by 254 nm light for 5 min resulted in a 69% or 70% decrease of the enzyme activity, respectively. Addition of higher concentrations of NAD^+ to the irradiated enzyme–analog mixture did not bring the recovery of the enzyme activity. This is a clear indication that the irradiation of the dehydrogenase–analog complex resulted in formation of covalent linkage between the enzyme and the analogs and thus the enzyme activity is permanently diminished.

The mechanism behind the photoinactivation caused by the NAD^+ analogs is believed to involve the formation of a complex of enzyme and NAD^+ analog through a reversible binding, which is subject to irradiation to form a covalent linkage between the analog and the enzyme. Irradiation of the complex of NAD^+ analog **1** or **2** and the dehydrogenase results in the generation of highly reactive nitrene intermediates that covalently bind to the amino acid side chains within the active site of the enzyme. Formation of such covalent bonds leads to the irreversible loss of the enzyme activity.

The formation of site-specific covalent bindings was supported by the fact that the enzyme inactivation was absent when the preirradiated NAD^+ analogs or irradiated dehydrogenase was used in the enzymatic assay. Such photoinduced inhibition cannot be recovered by addition of higher concentrations of natural coenzymes. In addition, photoinactivation of the enzyme activity by NAD^+ analog **1** or **2** can be reduced with the addition of the natural substrate NAD^+ prior to irradiation. The protection of the dehydrogenase from photoinactivation by natural substrate NAD^+ indicates that NAD^+ analogs **1, 2** and NAD^+ are competing for the same binding sites of the dehydrogenase.

CONCLUSION

The NAD^+ analogs **1** and **2** have been shown to fulfill the criteria required for photoaffinity labels based on the experiment data of the studies on inhibition and photoinactivation. The presence of a carbonyl functional group at the C-3 position of pyridine ring has proven to be essential for the retention of coenzyme activity for a photoaffinity label. Addition of a carboxylic functional group to the parent pyridine compound may significantly improve the reaction yield of the enzymatic base-exchange reaction. But it may also lead to an improper positioning of the pyridine moiety of the

analog, which, in turn, causes the loss of coenzyme activity in the study of binding sites of dehydrogenases.

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