Synthesis and Properties of Photoaffinity Labels for the Pyridine Dinucleotide Binding Site of NAD Glycohydrolase†

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ABSTRACT: Two new photoactive analogues of oxidized nicotinamide adenine dinucleotide (NAD^+) which are resistant to cleavage by NAD glycohydrolase were synthesized and characterized. The β-D-ribonucleotide ring of the nicotinamide riboside moiety of NAD^+ was replaced with a 2,3-dihydroxycyclopentane ring forming a carbocyclic dinucleotide analogue. Photoreactivity was achieved by the incorporation of an azido group at the 8-position of the adenosyl ring. The previously published synthesis of carbocyclic pyridine dinucleotide analogues [Slama, J. T., & Simmons, A. M. (1988) *Biochemistry 27*, 183] was modified by resolving the carbocyclic 1-aminoribose analogues and producing optically pure (+)-(1*S*)- or (−)-(1*R*)-4β-amino-2α,3α-dihydroxy-1β-cyclopentanemethanol. Each of these was converted to the corresponding carbocyclic nicotinamide 5′-nucleotide analogue and coupled with 8-azidoadenosine 5′-monophosphate. Two photoactive and isomeric NAD^+ analogues were thus prepared. 8-Azidoadenosyl carba-NAD is the analogue in which D-dihydroxycyclopentane is substituted for the D-ribose of the nicotinamide nucleoside moiety. 8-Azidoadenosyl pseudocarba-NAD contains the L-carbocycle in place of the D-ribotide ring. 8-Azidoadenosyl carba-NAD was shown to inhibit the NAD glycohydrolase from *Bungarus fasciatus* venom competitively with an inhibitor dissociation constant of 187 μM. 8-Azidoadenosyl pseudocarba-NAD was shown to inhibit the same enzyme competitively with a K_i of 73 μM. The superior NADase inhibitor, 8-azidoadenosyl pseudocarba-NAD, was characterized kinetically and shown to fulfill the criteria required of a specific active site directed photoaffinity probe. Irradiation of mixtures of the photoprobe and NAD glycohydrolase with short-wave ultraviolet light resulted in the rapid and irreversible loss of enzyme activity. No time-dependent enzyme inhibition was observed either with the photoprobe in the dark or on irradiation of solutions of the enzyme with analogues lacking the azido group. Rates of photoinactivation were slowed in the presence of the substrate NAD^+ and in the presence of nonphotoactive competitive inhibitors and were shown to saturate at low concentrations of photoprobe.

NAD glycohydrolases are a family of enzymes that catalyze the hydrolysis of oxidized nicotinamide adenine dinucleotide (NAD^+) to adenosine diphosphate ribose (ADP-ribose)[1] and nicotinamide. These enzymes are ubiquitously distributed and have been purified from both eukaryotic and prokaryotic sources. The NAD glycohydrolases are still poorly understood from the standpoint of their mechanisms of action, their mechanisms of regulation, and their biochemical function (Anderson, 1982; Price & Pekala, 1987). Important roles in cellular regulation have been suggested for NADases as well as for the related mono- and poly(ADP-ribosyl) transferases (Kaplan, 1966; Moss & Vaughan, 1988).

Several photoaffinity labels designed for pyridine dinucleotide binding sites have been described. The probes differ with respect to the site of substitution of the photoactivatable group in the dinucleotide analogue. The adenosyl moiety was derivatized with the photoactivatable azido group in 8-azidoadenosyl NAD (Koberstein, 1977) and in 2-azidoadenosyl NAD (Kim & Haley, 1990). The 3-carboxamido of the pyridinium ring of NAD^+ was replaced with a diazoacetoxymethyl group (Browne et al., 1971), an azide (Hixon & Hixon, 1973), or a diazirine (Standring & Knowles, 1980). Finally, an arylazido-β-alanine NAD has been synthesized (Chen & Guillory, 1977) in which a nitrophenyl azide is attached to a ribosyl hydroxyl by using a short spacer arm. Although these photoaffinity labels were successfully applied to the study of dehydrogenases, all of these photoprobes contain a labile pyridinium ribotide bond. This bond is subject to hydrolysis by the glycohydrolase activity of the target NADase. Since the dissociation constants of the products—ADP-ribose and nicotinamide—are considerably higher than that of the substrate dinucleotide, hydrolysis destroys the efficacy of these photoaffinity probes. ADP-ribosyl transferases are known to possess NAD glycohydrolase activity (Moss et al., 1976). Many of the transferases also catalyze efficient automodification in the absence of the acceptor cosubstrate. These reactions complicate the interpretation of any photoderivatization study using a photoprobe that is a transferase substrate.

Photoaffinity labels such as the carbocyclic pyridine dinucleotide analogues **1** and **2** (Figure 1) would be expected to be recognized by an NAD-specific active site and be resistant to hydrolysis. We recently developed a synthesis of the parent dinucleotides: carbocyclic NAD (**3**; carba-NAD) and the isomeric pseudocarbocyclic NAD (**4**; pseudocarba-NAD) (Slama & Simmons, 1988a). We further demonstrated that pseudocarba-NAD (**4**) is a potent competitive inhibitor for a variety of NAD glycohydrolases (Slama & Simmons, 1989).

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[1] Abbreviations: AppA, P^1,P^2-bis(5′-adenosyl)pyrophosphate; ADP-ribose, adenosine diphosphate ribose; BSA, bovine serum albumin; DMF, dimethyl formamide; Naproxen, (+)-(2*S*)-2-(6-methoxy-2-naphthyl)-propanoic acid; NADase, NAD glycohydrolase; NMN, nicotinamide 5′-mononucleotide; THF, tetrahydrofuran.

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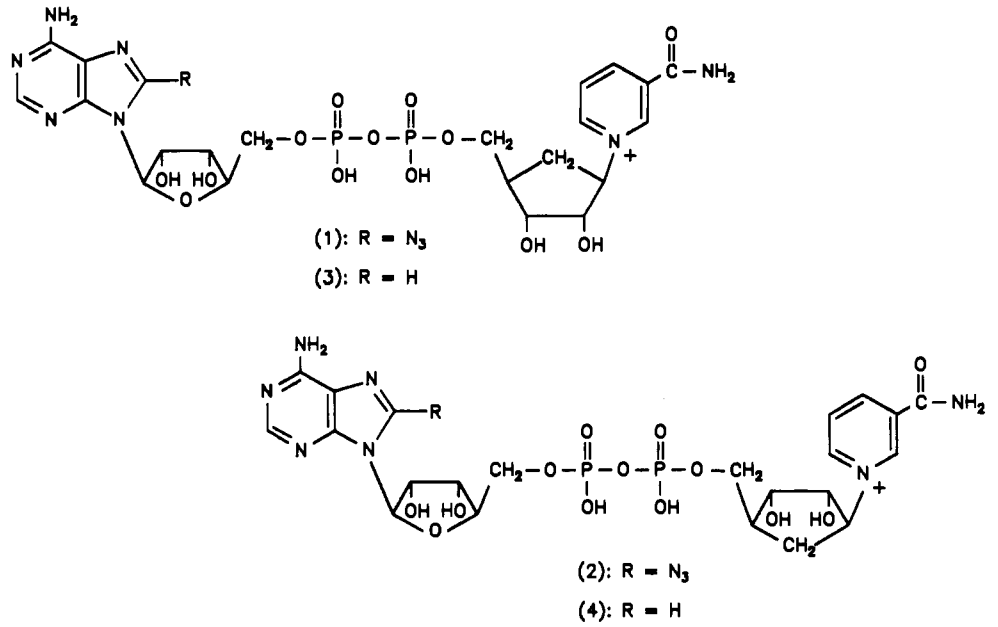

FIGURE 1: Structures of 8-N_3-carba-NAD (**1**), 8-N_3-pseudocarba-NAD (**2**), carba-NAD (**3**), and pseudocarba-NAD (**4**).

The photoprobes **1** and **2** should therefore be ideal photoaffinity labels for application to the study of NAD glycohydrolases and ADP-ribosyl transferases, combining the requisite reactivity, specificity, and stability.

The present paper describes the synthesis of two isomeric photoprobes (**1** and **2**), their chemical characterization, and their characterization as inhibitors of the *Bungarus fasciatus* venom NADase, a typical NAD glycohydrolase.

Experimental Procedures

General Methods. Proton NMR spectra were determined at 300 MHz and the chemical shifts reported in ppm from an internal standard of tetramethylsilane (TMS) (for organic solutions) or sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP) (for aqueous solutions).

Thin-layer chromatography was performed on 2.5 × 10 cm glass-supported 250 μM silica gel GHLF plates (for normal-phase chromatography) or on C-18 hydrocarbon-impregnated silica RPS-F plates (for reversed-phase chromatography)(Analtech Inc., Newark, DE). Preparative HPLC was performed by using a Waters Associates Delta-Prep 3000, equipped with a variable-wavelength ultraviolet detector and a preparative flowcell.

Fast-atom bombardment mass spectra were acquired on a Finnigan-MAT 212 mass spectrometer in combination with an INCOS data system. An Ion Tech saddle field atom gun operating at 8 kV was used with xenon gas. The ion source temperature was approximately 60 °C, and the accelerating voltage was 3 kV. Samples were dissolved in water (10 mg/mL), and 1 μL of the solution was applied to the copper probe tip. Thioglycerol (approximately 2 μL) was added and mixed thoroughly with the sample. The contribution from the matrix was subtracted from each spectrum.

Reagent grade THF was dried by distillation over sodium and benzophenone. Anhydrous DMF was purchased from Aldrich Chemical Co. (Milwaukee, WI) and transferred by syringe under an atmosphere of dry nitrogen. All other reagents and solvents were of the highest available purity and were used without further treatment.

Methyl (±)-4β-amino-2α,3α-dihydroxy-1β-cyclopentanecarboxylate hydrochloride was prepared and characterized as described in our previous work (Slama & Simmons, 1988).

Synthesis of the Diastereomeric Amides **5a** *and* **5b**. *N*-Hydroxysuccinimide (100 mmol, 11.51 g; Aldrich) and Naproxen (100 mmol, 23.03 g; Aldrich) were dissolved in 200 mL of THF. The solution was stirred magnetically under a blanket of nitrogen as diisopropylcarbodiimide (100 mmol, 15.7 mL) was added via syringe. Several minutes after completion of addition, a large amount of precipitate formed. The mixture was allowed to stir overnight at ambient temperature. The next morning precipitated diisopropylurea was removed by filtration. Solvent was removed in vacuo and the resulting residue crystallized from ethanol. The crystals were collected on a Buchner funnel and air dried to yield 29.01 g (88.72%) of white crystals: mp 115–118 °C. TLC (silica; 1% CH_3OH, 1% acetic acid in ethyl acetate) indicated a single major UV-positive component (R_f = 0.73).

A solution of the carbocyclic amino ester (10.55 g, 50 mmol) in 100 mL of anhydrous DMF was treated with the *N*-hydroxysuccinimidyl ester of Naproxen (18 g, 55 mmol, 110%) followed by triethylamine (7.7 mL, 110 mmol). A fine white precipitate formed almost immediately. The mixture was stirred overnight at ambient temperature under a blanket of nitrogen. The mixture was poured onto 500 mL of ice and water and a large amount of white precipitate formed. The ice was allowed to melt and the precipitate collected on a Buchner funnel and air dried to yield 18.17 g (94% yield) of a white solid. TLC (silica; 1% CH_3OH, 1% acetic acid in ethyl acetate) revealed a two-component mixture: R_f = 0.41 (**5a**) and R_f = 0.48 (**5b**).

Chromatographic Separation of **5a** *and* **5b**. The diastereomers **5a** and **5b** were separated by preparative HPLC using a Rainin Dynamax-60 A 8-μm irregular silica column (41.4-mm i.d. × 250 mm) equipped with a guard column (41.4-mm i.d. × 50 mm). The mixture of **5a** and **5b** (500 mg) was applied dissolved in chloroform (20 mL) and the chromatography developed isocratically at a flow rate of 80 mL/min with a solvent of 1% acetic acid and 2% CH_3OH in chloroform. Compound **5b** was the first of the two components to elute from the column at 11 min (880 mL), followed by **5a** at 12.5 min (1000 mL). Fractions were analyzed by HPLC, those containing either pure **5a** or **5b** were pooled, and the solvent was removed under reduced pressure. The solid isomers were crystallized from ethyl acetate and dried in vacuo to remove

traces of acetic yielding the following.

Methyl [1R]-4β-[(S)-(2-(6-methoxy-2-napthyl)-propionyl)amino]-2α,3α-dihydroxy-1β-cyclopentane-carboxylate (**5a**): mp 130–132 °C; $[\alpha]_D = -12.6°$ (c = 1, CH_3OH); ^1H NMR (CD_3OD) δ 1.42–1.48 (m, 1 H), 1.51 (d, J = 7 Hz, 3 H), 2.29–2.34 (m, 1 H), 2.78–2.81 (m, 1 H), 3.61 (s, 3 H), 3.75–3.83 (m, 2 H), 3.87 (s, 3 H), 4.07–4.16 (m, 2 H), 7.07–7.71 (aromatic, 6 H); mass spectrum, calcd for M^+ ($C_{21}H_{25}O_6N$) m/z 387, found m/z 387 (M^+).

Anal. Calcd for $C_{21}H_{25}O_6N$: C, 65.10; H, 6.50; N, 3.62. Found: C, 65.07; H, 6.55; N, 3.59.

Methyl [1S]-4β-[(S)-(2-(6-methoxy-2-napthyl)-propionyl)amino]-2α,3α-dihydroxy-1β-cyclopentane-carboxylate (**5b**): mp 183–185 °C; $[\alpha]_D = -17.4°$ (c = 1, CH_3OH). NMR (CH_3OD) δ 1.50 (d, J = 7 Hz, 3 H), 1.55–1.65 (m, 1 H), 2.40–2.45 (m, 1 H), 2.80–2.84 (m, 1 H), 3.66 (s. 3 H), 3.68–3.72 (m, 1 H), 3.75–3.78 (m, 1 H), 3.88 (s, 3 H), 4.03–4.09 (m, 2 H), 7.08–7.72 (aromatic, 6 H); mass spectrum, calcd for M^+ ($C_{21}H_{25}O_6N$) m/z 387, found m/z 387 (M^+).

Anal. Calcd for $C_{21}H_{25}O_6N$: C, 65.10; H, 6.50; H, 6.50; N, 3.62. Found: C, 65.34; H, 6.53; N, 3.53.

[1R]-4β-[(S)-(2-(6-Methoxy-2-napthyl)propionyl)-amino]-2α,3α-dihydroxy-1β-cyclopentanemethanol (**6a**). A solution of the carbocyclic amide methyl ester (**5a**, 1.0 g, 2.58 mmol) in 20 mL of dry THF was added dropwise to a solution of $LiBH_4$ (Alfa, 112 mg, 5.16 mmol, 200 mol %) in 12 mL of THF over 30 min. The reaction was stirred magnetically under a blanket of nitrogen at ambient temperature. Upon completion of the addition, TLC (silica; 1% acetic acid, 1% CH_3OH in ethyl acetate) indicated all of the methyl ester (R_f = 0.37) had been converted to the alcohol (R_f = 0.08). The reaction was quenched by the addition of 25 mL of methanolic HCl (prepared by the addition of 1 mL of acetyl chloride (14 mmol) to 25 mL of dry methanol). Solvent was evaporated in vacuo, the resulting residue twice dissolved in an additional 25 mL of methanolic HCl, and the solvent evaporated. A viscous oil (2.2 g) was recovered. This was dissolved in 50 mL of water and 10 mL of ethanol and extracted with three 50-mL portions of ethyl acetate. The organic phases were combined, dried briefly above sodium sulfate, and evaporated to yield 1.2 g of a white solid. Crystallization from ethyl acetate produced 0.83 g (89% yield) of a colorless solid: mp 183–186 °C.

A sample of analytical purity was obtained by flash column chromatography on C-18 reverse-phase silica gel (Toronto Research Chemicals, Downsview, Ont.). The sample (250 mg) was applied as a solution in CH_3OH (5 mL) to 20 g of C-18 packing equilibrated in 60% CH_3OH in H_2O. **6a** eluted into 2–4 bed volumes of 60% CH_3OH in H_2O. Evaporation followed by crystallization from CH_3OH gave 200 mg of colorless crystals: mp 188–189 °C; $[\alpha]_D = -12.16°$ (c = 0.51, CH_3OH); TLC (silica, 2% acetic acid, 10% CH_3OH in $CHCl_3$, R_f = 0.25; reversed-phase silica, 60% CH_3OH in H_2O, R_f = 0.75); ^1H NMR (CD_3OD) δ 1.01–1.11 (m, 1 H), 1.51 (d, J = 7 Hz, 3 H), 2.00–2.07 (m, 1 H), 2.11–2.21 (m, 1 H), 3.51 (dd, J = 2, 6 Hz, 2 H), 3.71–3.76 (m, 2 H), 3.78–3.82 (m, 1 H), 3.88 (s, 3 H), 4.05–4.12 (m, 1 H), 7.07–7.71 (aromatic, 6 H); mass spectrum, calcd for M^+ ($C_{20}H_{25}O_5N$) m/z 359, found m/z 359 (M^+).

Anal. Calcd for $C_{20}H_{25}O_5N$: C, 66.83; H, 7.01; N, 3.90. Found: C, 66.48; H, 6.89; N, 3.91.

[1S]-4β-[(S)-(2-(6-Methoxy-2-napthyl)propionyl)-amino]-2α,3α-dihydroxy-1β-cyclopentanemethanol (**6b**) was prepared identically to **6a** except that the starting methyl ester (**5b**) was not readily soluble in THF. It was necessary to heat the amide in THF (1.0 g in 50 mL) to achieve a solution and the solution in the dropping funnel was periodically warmed with a heat gun to maintain solubility over the course of the addition. Crystallization from ethyl acetate and ethanol resulted in the isolation of 0.87 g (94% yield) of colorless crystals: mp 138–141 °C; TLC (silica, 1% CH_3OH, 1% acetic acid in ethyl acetate, R_f = 0.08).

A sample of analytical purity was obtained by chromatography identical with that for **6a**, followed by crystallization from CH_3OH and H_2O: mp 141–143 °C; $[\alpha]_D = +3.53°$ (c = 0.51, CH_3OH); TLC (silica, 2% acetic acid, 10% CH_3OH in $CHCl_3$, R_f = 0.25; reversed-phase silica, 60% CH_3OH in H_2O, R_f = 0.73); ^1H NMR (CD_3OD) δ 1.18–1.25 (m, 1 H), 1.50 (d, J = 7 Hz, 3 H), 2.00–2.10 (m, 1 H), 2.20–2.32 (m, 1 H), 3.55 (dd, J = 4, 5 Hz, 2 H), 3.65 (t, J = 6 Hz, 1 H), 3.72–3.76 (m, 2 H), 3.87 (s, 3 H), 4.03–4.10 (m, 1 H), 7.07–7.71 (aromatic, 6 H); mass spectrum, calcd for M^+ ($C_{20}H_{25}O_5N$) m/z 359, found m/z 359 (M^+).

Anal. Calcd for $C_{20}H_{25}O_5N \cdot {}^1/_2H_2O$: C, 65.20; H, 7.11; N, 3.80. Found: C, 65.14; H, 7.19; N, 3.71.

(−)-[1R]-4β-Amino-2α,3α-dihydroxy-1β-cyclopentane-methanol (**7a**). **6a** (2.63 g, 7.33 mmol) was suspended in 250 mL of 1 M HCl and refluxed under nitrogen. After 48 h, analysis using TLC [silica gel; 1-butanol–acetic acid–water (5:2:3)] indicated the presence of a single UV-positive component isographic with Naproxen (R_f = 0.85) and a single ninhydrin-positive component isographic with the amine triol (R_f = 0.41). The reaction was allowed to cool to ambient temperature and Naproxen precipitated from solution. Ethanol (25 mL) and ethyl acetate (100 mL) were added and the mixture was transferred to a separatory funnel. The aqueous phase was extracted with two additional 100-mL portions of ethyl acetate and evaporated in vacuo to yield 1.33 g (99% yield) of the carbocyclic amine hydrochloride. To isolate the amine free from salt, the residue was dissolved in 20 mL of water and applied to a column of Bio-Rad AG 50W-X8, H^+ form (40 mL). The resin was washed with water until the effluent was free from chloride, and then the amine was eluted into 1 M NH_4OH. After evaporation, a colorless oil was recovered (1.08 g, 100% yield): $[\alpha]_D = -6.3°$ (c = 1.24, H_2O) [lit.: $[\alpha]_D = -10.3°$ (c = 1.52, H_2O) (Arita et al., 1983); $[\alpha]_D = -10°$ (c = 0.5, H_2O) (Sicsic et al., 1987)].

(+)-[1S]-4β-Amino-2α,3α-dihydroxy-1β-cyclopentane-methanol (**7b**) was prepared identically with **7a** resulting in the production of a colorless oil in quantitative yield: $[\alpha]_D = +9.5°$ (c = 1.44, H_2O). [lit.: $[\alpha]_D = +9°$ (c = 0.5, H_2O) (Sicsic et al., 1987)].

D-*Carbanicotinamide 5′-mononucleotide* (carba-NMN, **8a**) was prepared from **7a** according to the procedure of Slama and Simmons (1988a).

L-*Carbanicotonamide 5′-mononucleotide* (pseudocarba-NMN, **8b**) was prepared from **7b** according to the procedure of Slama and Simmons (1988a).

8-Azidoadenosine 5′-monophosphate was prepared according to the procedure of Czarnecki et al. (1979).

8-Azidoadenosyl Carbanicotinamide Adenine Dinucleotide (**1**, *8-N_3-carba-NAD*). 1′,2′-Di-*O*-acetylcarbanicotinamide mononucleoside 5′-phosphate was prepared from **8a** by the method of Slama and Simmons (1988a). Coupling of the nucleotides was accomplished with the Michelson procedure, which utilizes diphenyl phosphorochloridate as a coupling reagent (Michelson, 1964). 8-N_3-AMP (175 mg, 0.45 mmol) was dissolved in 4.5 mL of anhydrous methanol. Trioctylamine (197 μL, 0.45 mmol) was added and the mixture stirred magnetically. A solution formed after 10 min. Methanol was

evaporated, and the residue was dried three times by adding 3 mL of dry DMF and evaporating the solvent in vacuo. The trioctylammonium salt was dissolved in 2.0 mL of dry dioxane and diphenyl phosphorochloridate (140 μL, 0.675 mmol, 50% excess) added followed by tributylamine (241 μL, 0.90 mmol, 2-fold excess). A homogeneous solution formed after several minutes of stirring at ambient temperature and under nitrogen. After 2 h, TLC (silica; ethanol/1 M NH_4OAc, 5:2) indicated the reaction was complete by consumption of 8-N_3-AMP (R_f = 0.44). Solvent was evaporated, and ether was added (12 mL) to precipitate the activated 8-N_3-AMP. After the mixture stood for 15 min at 0 °C, ether was decanted and solvent removed from the residual gum by twice adding 3 mL of dry dioxane and removing the solvent in vacuo. Activated 8-N_3-AMP was dissolved in 2.0 mL of anhydrous DMF. Diacetylcarbanicotinamide mononucleotide (280 mg, 0.67 mmol, 50% excess) was dissolved in 5.0 mL of DMF and added to the activated nucleotide, followed immediately by 16 mL of dry pyridine. The flask was capped with a septum, purged with N_2, and wrapped in Al foil to protect the contents from light. The reaction was stirred for 72 h, then solvent was evaporated, and ether (20 mL) was added to precipitate the products. After the mixture stood for 30 min at 0 °C, ether was decanted. Analysis of the precipitate by TLC (silica; ethanol/1 M NH_4OAc, 5:2) showed a single major component (R_f = 0.39), a secondary spot isographic with diacetylcarbanicotinamide mononucleotide (R_f = 0.11), and only a trace of 8-N_3-AMP (R_f = 0.47). The precipitate was dissolved in 20 mL of ice-cold methanolic ammonia (3 N). After the mixture was stirred for 2 h in an ice-water bath, TLC (reversed-phase silica; 50 mM KH_2PO_4, pH 3.5/acetonitrile, 90:10) showed complete conversion of the major component (R_f = 0.28) to a new more polar material (R_f = 0.67). A secondary spot appeared, which was isographic with carbanicotinamide mononucleotide (R_f = 0.58). The deprotection reaction was stirred for a total of 3 h, when solvent was removed under reduced pressure. The last traces of ammonia were removed by repeated addition of methanol followed by removal of solvent in vacuo. 8-N_3-carba-NAD was purified by ion-exchange chromatography on Bio-Rad AG1-X 2 anion exchange resin, 100–200 mesh, acetate form (80 mL, 47 × 1.5 cm). The dinucleotide eluted from the resin in a linear gradient formed between 0 and 2 M acetic acid (400 mL each). The dinucleotide appeared in the last third of the gradient. Appropriate fractions were combined and lyophilized to give 100 mg (31% yield) of a white powder. The lyophilizate was found to be homogeneous when examined by two different HPLC systems. Analysis using reversed-phase HPLC was performed on a DYNAMAX 8-μm C-18 column (4.6 × 250 mm, Rainin, Woburn, MA) eluted isocratically at 1.0 mL/min with 20 mM NaH_2PO_4, which was adjusted to pH 6.0 by using 12 M tetrabutylammonium hydroxide (retention time 19.6 min). Ion-exchange chromatography utilized a 10-μm RSIL AN anion-exchange column (4.6 × 250 mm, Alltech, Deerfield, IL) developed isocratically with 250 mM KH_2PO_4, pH 3.5, at 1.0 mL/min (retention time 6.2 min). The electronic absorption spectrum of 8-N_3-carba-NAD, **1** (50 mM KH_2PO_4, pH 7.0), exhibits a maximum at 274 nm (ϵ_{274} = 11.86 × 10^3 M^{-1}/cm^{-1}). Upon reduction at pH 10 [0.5 M Tris·HCl, 0.5 M ethanol, and yeast alcohol dehydrogenase (Sigma)], a second absorption appears at 357 nm and a shift occurs in the first band to 278 nm (ϵ_{357} = 4.6 × 10^3 M^{-1}/cm^{-1}; A_{278}/A_{357} = 3.17). ^1H NMR (D_2O): signals assigned to the 2,3-dihydroxycyclopentane ring, δ 2.16–2.27 (m, 1 H), 2.40–2.46 (m, 1 H), 2.60–2.70 (m, 1 H); signals assigned to the pyridinium ring, δ 8.18 (dd, J = 6, 8 Hz, 1 H), 8.86 (d, J = 8 Hz, 1 H), 9.14 (d, J = 6 Hz, 1 H), 9.37 (s, 1 H); signal assigned to the adenosyl anomeric proton, δ 5.90 (d, J = 5 Hz, 1 H); signal attributed to the adenine, δ 8.23 (s, 1 H). The remaining five dihydroxycyclopentane protons and five ribosyl protons appear in the region between 4.0 and 5.1 ppm as a group of complex multiplets, together with the HDO signal. Fast atom bombardment mass spectrum calcd for M^+ ($C_{22}H_{28}N_{10}O_{13}P_2$) m/z 702, found m/z 703 ($[MH]^+$).

8-Azidoadensoyl pseudocarbocyclic nicotinamide adenine dinucleotide (**2**, 8-N_3-pseudocarba-NAD) was prepared by using a procedure identical with that employed for its diastereomer **1**, except using the carba-NMN enantiomer **8b**. It was indistinguishable from its diastereomer in both the reversed-phase (retention time 19.6 min) and anion-exchange (retention time 6.2 min) HPLC systems. At pH 7.0 (50 mM KH_2PO_4) the electronic absorption spectrum exhibits a maximum at 274 nm (ϵ_{274} = 11.81 × $10^3/M^{-1}/cm^{-1}$). ^1H NMR (D_2O): signals attributed to the 2,3-dihydroxycyclopentane ring, δ 2.19–2.30 (m, 1 H), 2.40–2.50 (m, 1 H), 2.67–2.77 (m, 1 H); pyridinium signals, δ 8.16 (dd, J = 6, 8 Hz, 1 H), 8.84 (d, J = Hz, 1 H), 9.14 (d, J = 6 Hz, 1 H), 9.37 (s, 1 H); signal assigned to the adenosyl anomeric proton, 5.89 (d, J = 5 Hz, 1 H); signal attributed to the adenosine proton, 8.22 (s, 1 H). The region between 4.0 and 5.1 ppm contains the remaining dihydroxycyclopentane protons and the ribosyl protons, which appear as complex multiplets along with the HDO absorption. Fast atom bombardment mass spectrum calcd for M^+ ($C_{22}H_{28}N_{10}O_{13}P_2$) m/z 702, found m/z 703 ($[MH]^+$).

NAD glycohydrolase from B. fasciatus venom was purified to homogenity according to the three-step procedure of Yost and Anderson (1981), as described in our previous publication (Slama & Simmons, 1988).

NAD Glycohydrolase Assay. The rate of hydrolysis of NAD^+ by the venom glycohydrolase was measured radiometrically by measuring the release of [carbonyl-^{14}C]nicotinamide from [carbonyl-^{14}C]NAD^+ (Slama & Simmons, 1988). The standard assay was conducted for 30 min at 37 °C in 0.3 mL of 33 mM potassium buffer, pH 7.5, containing 50 μM [carbonyl-^{14}C]NAD^+ (Amersham; 50000 dpm) and enzyme (0.06 milliunit). A unit of NADase activity is that quantity of enzyme which catalyzes the hydrolysis of 1 μmol of NAD^+/min.

Photoinactivation of B. fasciatus venom NAD glycohydrolase by **1** or **2** was conducted at 0 °C at pH 7.5 (33 mM phosphate buffer containing 33 μg/mL bovine serum albumin). A solution of the NADase and photoprobe (150 μL) was incubated 5 min at room temperature in a spot well plate, then cooled to 0 °C on ice, and irradiated with a short-wave (254 nm) mineral lamp (Model UVG-11, UVP Inc., San Gabriel, CA) held at a distance of 20 cm.[2] Aliquots (10 μL) were removed at intervals and diluted 100:1 into 5 mM phosphate buffer, pH 7.5, containing 0.1 mg/mL BSA. The remaining enzyme activity was determined by assay under standard conditions.

Protection of the NADase from photoinactivation by **1** or **2** in the presence of the substrate NAD was demonstrated by

[2] The rates of photoinactivation are sensitive to the intensity of the ultraviolet light. When careful measurement of the rates of photoinactivation was desired, the light intensity was decreased by increasing the distance between the lamp and the reaction or by substituting an old and less intense UV lamp. Our reference to *conditions of low illumination* indicates that the intensity of the light was so adjusted to produce a $t_{1/2}$ for inactivation on the order of 5 min.

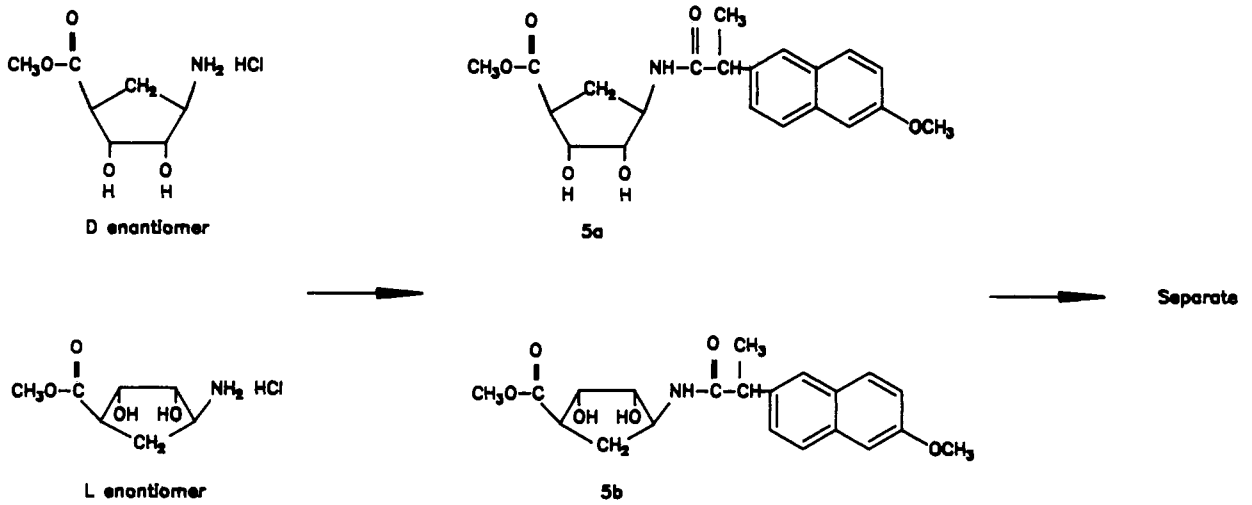

FIGURE 2: Preparation of the diastereomeric Naproxen amides **5a** and **5b** from methyl (±)-4β-amino-2α,3α-dihydroxycyclopentanecarboxylate.

adding NAD (10–200 μM) to a 150-μL reaction that was cooled to 0 °C with ice on a spot well plate. The reaction contained containing 20 μM photoprobe, 46 mM phosphate buffer, pH 7.5, and 33 μg of BSA. The reaction was initiated by the addition of 0.02 unit of the NADase and immediately irradiating the mixture with a UV light fixed at a distance of 20 cm. After 60 s a 10-μL aliquot was removed and diluted 100:1 into 5 mM phosphate buffer, pH 7.5, containing 0.1 mg/mL BSA. The remaining enzymatic activity was measured under standard conditions.

Analysis of Enzyme Inhibition Data. Kinetic parameters for competitive inhibitors were determined by using the nonlinear regression analysis developed by Cleland (1979) using a translation of the programs for the IBM PC and compatibles (obtained from Dr. R. E. Viola, Department of Chemistry, The University of Akron, OH). Each data set was fit to equations describing linear competitive, noncompetitive, and uncompetitive inhibition. The best description of the data was chosen according to the criteria set forth by Cleland for the evaluation of a successful fit.

Results and Discussion

The parent carbocyclic NAD analogues, carba-NAD (**3**) and pseudocarba-NAD (**4**), were synthesized by chemically coupling adenosine 5′-monophosphate with the racemic carbanicotinamide 5′-nucleotide (**8a** and **8b**). The isomeric dinucleotides **3** and **4** were therefore first obtained as their equimolar mixture. The individual purified isomers were subsequently prepared by using preparative reversed-phase HPLC (Slama & Simmons, 1988a, 1989). We have previously reported the successful coupling of racemic carbanicotinamide mononucleoside 5-phosphate (**8a,b**) with an activated 8-N_3-AMP, resulting in the synthesis of a mixture of the two diastereomeric photolabels **1** and **2** (Slama & Simmons, 1990a). We were unable, however, to separate the mixture of the substituted dinucleotides **1** and **2**. In order to obtain **1** and **2** pure, the synthesis was modified and the precursor carbocyclic sugar (±)-**7** resolved.

Resolution of the Carbocyclic Aminoribose Analogues and Synthesis of Photoprobes **1** *and* **2**. After experimentation with the separation of diastereomeric derivatives of the various precursors of **7**, we found that when (±)-4β-amino-2α,3α-dihydroxycyclopentane-1β-carboxylate was coupled with the readily available and inexpensive chiral acid Naproxen the diastereomeric amides **5a** and **5b** (Figure 2) were easily separated chromatographically. The scale and efficiency of the separation was increased by using preparative HPLC. Isocratic runs employing a 50 × 250 mm silica column were capable of achieving a baseline separation of the diastereomeric amides in 30 min. Rechromatography of purified **5a** and **5b** under analytical conditions reveals that each fraction contains less than 1% of the other diastereomer.

Synthesis of the individual free amines **7a** and **7b** was completed as shown in Figure 3. The absolute configuration of the amines **7a** and **7b** was established by measuring the specific rotation of each enantiomer in water. Previous work (Arita et al., 1983; Sicsic et al., 1987) established that the levorotatory (−)-enantiomer corresponds to the configuration of (−)-(1*R*)-**7a**, the carbocyclic analogue of 1-amino-D-ribofuranose (Figure 3).

Each enantiomer of 4β-amino-2α,3α-dihydroxy-1β-cyclopentanemethanol (**7a** or **7b**) was individually converted to the carbocyclic dinucleotides **1** and **2** by using a slight variation of our published procedure (Figure 3) (Slama & Simmons, 1988).

Spectroscopic Properties of the Photoaffinity Labels. The ultraviolet absorption spectra of 8-N_3-carba-NAD (**1**) show maxima at 230 nm and at 274 nm. Upon reduction to 8-N_3-carba-NADH by yeast alcohol dehydrogenase, a new absorption maxima at 357 nm is produced. The position of the new absorption maxima, at a higher wavelength than the typical dihydronicotinamide absorption (at 340 nm in N_3-NADH; Koberstein, 1976), is the result of the substitution of the furanose oxygen with methylene (Slama & Simmons, 1988). The diastereomeric photoprobe **2** has an identical absorption spectra but cannot be reduced enzymatically by yeast alcohol dehydrogenase.

The absorption spectra for 8-N_3-carba-NAD and 8-N_3-pseudocarba-NAD both show differences between acidic and neutral pH values (Figure 4). Although the position of the absorption maximum at 274 nm is invariant, the intensity of the band increases significantly at low pH. A 15% increase in the intensity of the chromophore is associated with lowering of the pH from 7 to 2. This hyperchromicity is associated with protonation of the adenosyl N-1, and the disruption of intramolecular association. The existence of the hyperchromicity suggests that there is less intramolecular association in the dinucleotide analogues **1** and **2** at neutral and basic pH than that present in 8-N_3-NAD, which shows 30% hyperchromicity at this same band upon acidification (Koberstein, 1976).

Chemical Properties of the Photoaffinity Labels. Each of the photoprobes was isolated as an amorphous, stable solid following ion-exchange chromatography and lyophylization. The azides were stable to normal fluorescent room light during

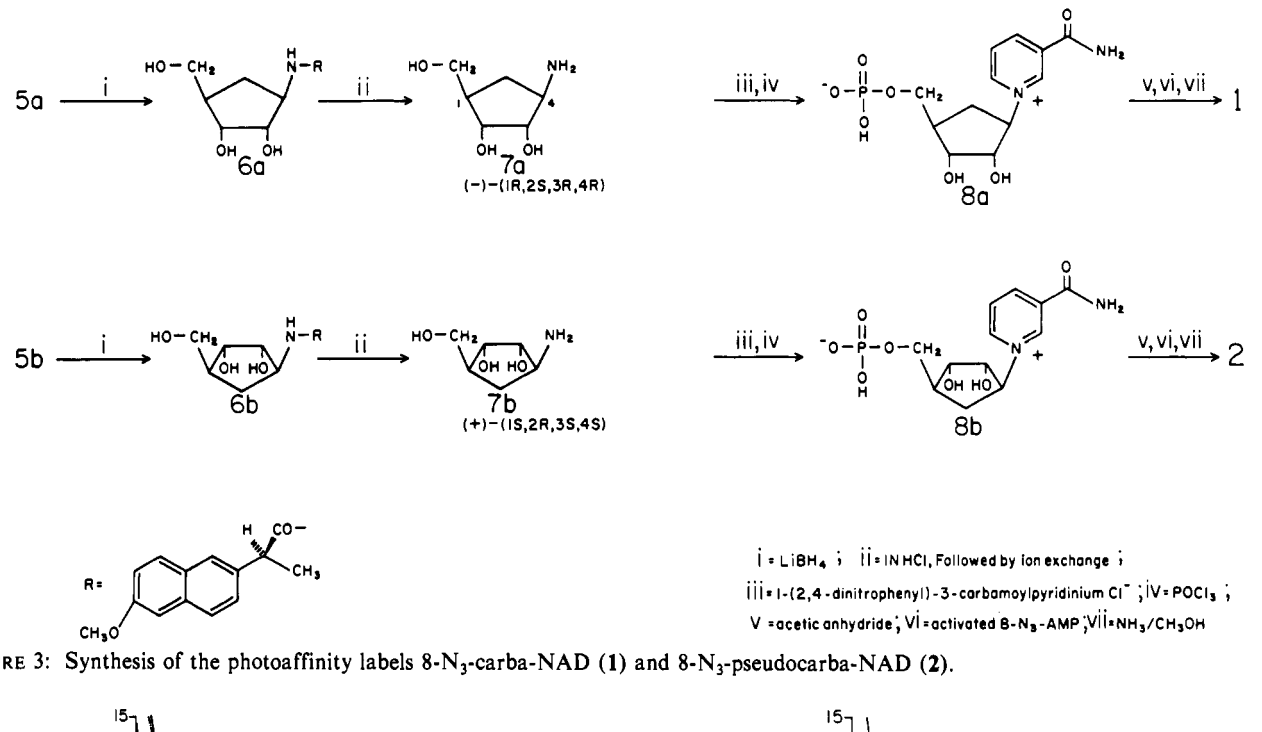

FIGURE 3: Synthesis of the photoaffinity labels 8-N_3-carba-NAD (**1**) and 8-N_3-pseudocarba-NAD (**2**).

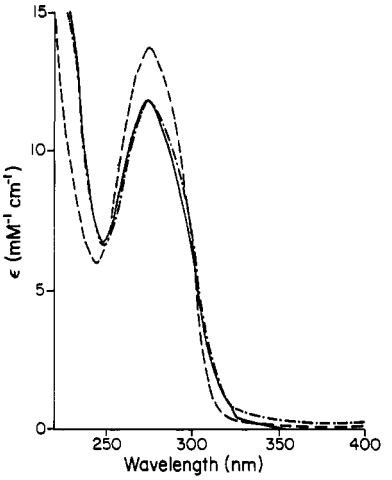

FIGURE 4: Absorption spectra of 8-N_3-pseudocarba-NAD (**2**): neutral (—) in 50 mM KH_2PO_4, pH 7; acid (---) in 10 mM HCl, pH 2; basic (···) in 50 mM Tris, pH 10.

purification, as a solid powder, and in dilute aqueous solution. On irradiation with short-wave ultraviolet light, the absorption spectrum changes (Figure 5) due to photolysis of the azide. The absorption maximum at 274 nm disappears, and a new lower intensity absorption appears at 265 nm. Similar changes are produced in the absorption spectra of 8-N_3-AMP and 8-N_3-NAD on irradiation with short-wave ultraviolet light, interpreted as the result of production of the 8-hydroxylamino nucleotide (Koberstein, 1976).

Inhibition of NAD Glycohydrolase. In a previous publication we investigated the ability of the parent carbocyclic dinucleotides to competitively inhibit the venom NAD glycohydrolases (Slama & Simmons, 1989). We determined to our surprise that carba-NAD (**3**) was ineffective as an inhibitor but that the diastereomeric pseudocarba-NAD (**4**) was a potent linear competitive inhibitor of the *B. fasciatus* venom NAD glycohydrolase with a dissociation constant of 35 μM. This surprising stereochemical relationship is true for several NAD glycohydrolases. Although it cannot yet be explained, it offers the possibility of selectively inhibiting the sensitive NAD glycohydrolase activity using **4** even in the presence of other interfering pyridine dinucleotide binding sites.

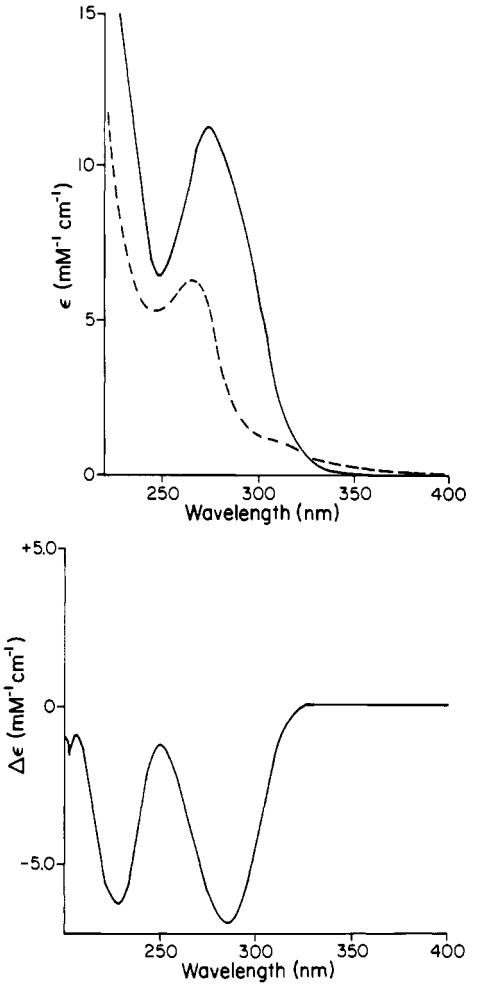

FIGURE 5: Ultraviolet irradiation of 8-N_3-pseudocarba-NAD (**2**) in 50 mM KH_2PO_4 buffer at pH 7. Upper: before irradiation (—); after photoreaction was complete (---). Lower: difference spectrum.

Addition of 8-N_3-pseudocarba-NAD (**2**) to assays of the *B. fasciatus* venom NAD glycohydrolase inhibits the enzyme-catalyzed NAD^+ hydrolysis (data not shown). The data support a linear competitive mechanism for inhibition, with

Table I: Photoinactivation of *B. fasciatus* Venom NAD Glycohydrolase

conditions of irradiation[a]	enzyme act. (milliunits/ 150 μL)[b]	enzyme act. (rel)
enzyme alone: in the dark	17.7	100%
enzyme alone: irradiated	17.7	100%
enzyme and **2**: in the dark	17.7	100%
enzyme and **2**: irradiated	1.98	11%
enzyme and preirradiated **2**:[c] irradiated	14.9	84%
enzyme and **4**: irradiated	17.7	100%
enzyme and 8-N_3-AMP: irradiated	7.3	41%
enzyme and 8-N_3-adenosine: irradiated	15.27	86%

[a] A solution of the NADase in 33 mM phosphate buffer (150 μL), pH 7.5, containing 20 μM nucleotide was placed in a cavity of a porcelain spot well plate for 5 min at ambient temperature, cooled to 0 °C (on ice), and irradiated for 2 min with a short-wave ultraviolet lamp held at a distance of 30 cm. [b] Aliquots (10 μL) from each solution were removed and diluted 100-fold and 50 μL of the dilution was assayed for NADase activity as described under Experimental Procedures. [c] Preirradiation of **2** was conducted on a 0.1 mM solution for 60 min in phosphate buffer, pH 7.5, until the electronic absorption spectrum indicated that the conversion to photoproducts was complete. A portion of this solution was immediately transferred to the spot well plate, enzyme was added, and the mixture irradiated under standard conditions.

an inhibitor dissociation constant, K_i, of 73 (±3) μM. 8-N_3-carba-NAD (**1**) was also found to be a linear competitive inhibitor of the venom NAD glycohydrolase, with a higher inhibitor dissociation constant, K_i, of 187 (±5) μM (data not shown). Apparently, substitution at the adenosyl 8-position increases the dissociation constant in the pseudocarba-NAD series slightly (i.e., **2** versus **4**) but substantially decreases the dissociation constant in the carba-NAD series (i.e., **1** versus **3**). No time-dependent loss in enzyme activity was observed on incubation of the NADase with either **1** or **2** in the dark.

Photoinactivation of B. fasciatus Venom NAD Glycohydrolase by 8-N_3-carba-NAD (**1**). Irradiation of the mixture of venom NADase and the photoactive dinucleotide **1** with short-wave ultraviolet light caused the rapid loss of enzymatic activity. The rate of photoinactivation followed pseudo-first-order kinetics through the first 70% of the reaction.[2] To demonstrate that a specific active site mediated photoinactivation was occurring, protection studies were done by using the substrate NAD+. Addition of NAD+ to mixtures of the enzyme and photoprobe prior to irradiation resulted in decreased photoinhibition of the NAD glycohydrolase. The protection afforded by increasing concentrations of NAD+ saturated at 28 ± 13 μM, a concentration in the vicinity of the known substrate K_m [35 μM, Slama and Simmons (1988a); 14 μM, Yost and Anderson (1981)].

Photoprobes **1** and **2** are efficient competitive inhibitors and are effective photoaffinity labels for the venom NADase. However, neither **2** nor its parent dinucleotide **4** are recognized by dehydrogenases. The isomeric photoprobe **2** is therefore expected to be a more selective photoprobe for the binding sites of sensitive NAD glycohydrolases. Since several of our anticipated applications will involve photolabeling studies in tissues, cells, or crude cellular homogenates, the selectivity of photoprobe **2** for the binding sites of NADases is an advantage. The obvious stereochemical difference between **2** and NAD as well as our anticipated applications of **2** as a selective photolabel prompted us to characterize the specificity of this photoaffinity label in detail.

Photoinactivation of the B. fasciatus NADase by the Isomeric 8-N_3-pseudocarba-NAD (**2**). Irradiation of the mixture of venom NADase and the photoactive dinucleotide **2** with short-wave ultraviolet light causes a rapid loss of enzymatic activity (Table I). The enzyme was not inactivated by irradiation in buffer alone, in solutions containing the nonphotoactive dinucleotide **4**, or in solutions where the azide group of photoprobe **2** had been destroyed by preirradiation. The venom NADase was further demonstrated to be stable and uninhibited on incubation with photoprobe **2** in the dark. Irradiation of the enzyme with 8-N_3-AMP resulted in significantly less inactivation than the irradiation in the presence of dinucleotide **2**. 8-Azidoadenosine was still less effective in the photoinactivation.

Table II: Rates of Inactivation of the Venom NADase by **2** in the Presence of Effectors[a]

conditions; NADase and **2** and effector[b]	$t_{1/2}$ for inactivation	K_i for effector[c]
adenosine (2 mM)	3.2 min	no inhibition
ADP-ribose (2 mM)	5.4 min	0.4 mM
AppA (1 mM)	5.5 min	unknown (ADP = 0.5 mM)
pseudocarba-NAD (4, 2.28 mM)	∞ no inactivation	0.035 mM[d]

[a] A solution of the NADase (150 μL) in 33 mM phosphate, pH 7.5, containing 20 μM **2** and the specified concentration of effector was equilibrated and irradiated with ultraviolet light under standard conditions (Table I, footnote a). Aliquots were removed at 2-min intervals, diluted 100-fold, and assayed for NADase activity immediately. The half-life of enzymatic activity was determined from plots of log (rel act.) verses time. [b] The concentrations of the effectors were selected so that all the solutions had equal absorbance at 259 nm. [c] Yost and Anderson (1982). [d] Slama and Simmons (1988a).

The rate of inactivation under conditions of low illumination[2] was pseudo-first-order through at least the first 85% of the reaction. Rates of photoinactivation slowed 2-fold in the presence of 100 μM isonicotinic acid hydrazide, a linear noncompetitive inhibitor with respect to NAD+ (K_i = 10 μM; Yost & Anderson, 1982), but not in the presence of 100 μM 3-acetylpyridine, a substituted pyridine that is a poor NADase inhibitor (K_i = 19 500 μM).

Addition of NAD+ to mixtures of the enzyme and photoprobe **2** prior to irradiation resulted in decreased photoinhibition of the NAD glycohydrolase. The protection afforded by increasing concentrations of NAD+ saturated at 13 ± 6 μM, a concentration in the vicinity of the known substrate *Km* [35 μM, Slama and Simmons, (1988a); 14 μM, Yost and Anderson (1981)]. To further demonstrate that 8-N_3-pseudocarba-NAD (**2**) is inactivating the enzyme through a specific reaction at the active site, rates of inactivation were measured in the presence of either adenosine or competitive inhibitors of the enzyme (Table II). The product of the reaction, ADP-ribose, slowed the rate of photoinactivation by about a factor of 2, as did the dinucleotide AppA. The nonphotoactive dinucleotide pseudocarba-NAD (**4**), a potent competitive inhibitor of the glycohydrolase, offered complete protection against photoinactivation.

Photoinactivated enzyme was not reactivated after removal of low molecular weight effectors by gel filtration. The effects of azide photodecomposition products, which as dinucleotide analogues might inhibit the NADase, were already shown to be largely removed from the enzyme by dilution (Table I). We conclude therefore that the inhibition of NAD glycohydrolase that we observe is irreversible.

The mechanism of specific photoinactivation by a competitive inhibitor such as **2** is expected to involve reversible binding of the inhibitor to the active site as a first step. The strength of this binding is governed by the inhibitor dissociation constant K_i, which we have determined to be 73 μM for **2**. The rates of specific photoinactivation should therefore increase with increasing concentrations of **2** but saturate as the concentration of the photoprobe approaches its K_i. To demon-

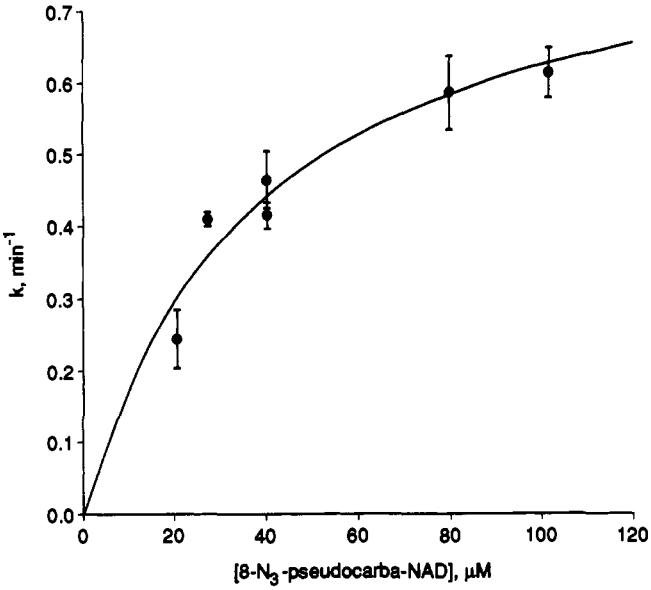

FIGURE 6: Rates of photoinactivation versus concentration of photoprobe **2**. Conditions for the photoinactivation are described in Table II, footnote a. Data were fit to the equation $k = k_i[2]/(K_{diss} + [2])$ by using a nonlinear regression analysis and values for k_i and K_{diss} were computed. The solid line represents the theoretical line calculated by using the empirically determined values of k_i and K_{diss}. To establish that the increasing optical absorbance of the solution did not affect the rate of photoinactivation, rates of inactivation in the presence of 20 μM **2** were measured in the presence and absence of 80 μM adenosine. Rates of photoinactivation for these two cases were the same to within experimental error (i.e., ±10%).

strate saturation effects with photoprobe **2**, the enzyme was photoinactivated by using concentrations of **2** from between 10 and 100 μM. Solutions of the NADase and photoprobe **2** were irradiated and the rate of enzyme inactivation was measured by determining the pseudo-first-order rate constant for inactivation. Plots of k_{app}, the pseudo-first-order rate constant for enzyme inactivation, versus the photoprobe concentration are hyperbolic (Figure 6) and when fit to the equation

$$k_{app} = k_{cat}[2]/\{K_{diss} + [2]\}$$

define a $K_{diss\,app}$ of 38 (±11) μM. Given the errors inherent in the rate measurement, this value is in agreement with the inhibitor dissociation constant determined in the dark.

The dinucleotide analogue 8-N_3-pseudocarba-NAD (**2**) is a potent linear competitive inhibitor of the venom NADase. Irradiation of mixtures of the photoprobe and the enzyme result in the irreversible loss of enzyme activity. Effective enzyme inactivation requires both light and the presence of an azido group in an intact pyridine dinucleotide. The absence of substantial enzyme inactivation by using preirradiated label eliminates the possibility that inactivation is the result of the production of a long-lived and reactive photoproduct.

The rates of NADase photoinactivation by **2** were shown to follow pseudo-first-order kinetics. We could therefore obtain information about the specificity of photoinactivation by comparing these relative rates. Photoinhibition is decreased in the presence of the substrate NAD^+, and this protection shows the expected saturation near the substrate K_m. Rates of photoinactivation were shown to be lowered in the presence of nonphotoactive inhibitors. The efficacy of the inhibitor in protecting the enzyme from photoinactivation correlates roughly with the inhibitor dissociation constant. The rates of photoinactivation further are shown to saturate at concentrations of the photoprobe close to the K_i of the inhibitor measured in the dark. The dinucleotide analogue 8-N_3-pseudocarba-NAD (**2**) has therefore been shown to fulfill the criteria required of a photolabel specific for the pyridine nucleotide binding site of a typical NAD glycohydrolase. It will now be applied to photoaffinity studies designed to elucidate the active-site structure of NAD glycohydrolases and related ADP-ribosyl transferases.

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