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Photodependent Inhibition of Bovine Spleen NAD^+Glycohydrolase by 8-Azido Carbocyclic Analogs of NAD^+

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Carba-NAD^+ and pseudocarba-NAD^+, and their 8-azidoadenosyl derivatives, were found to be good competitive inhibitors of calf spleen NAD^+glycohydrolase. The 8-azido compounds, tested as photoaffinity labels, inhibited the enzyme in a light- and time-dependent manner; this inhibition could be prevented by 3-aminopyridine adenine dinucleotide (n^3PdAD^+), a competitive inhibitor of NAD^+glycohydrolase. Irradiation in the presence of the [^{3}H]-labeled 8-azido-carba-NAD^+ derivative resulted in an irreversible incorporation of the radioactivity into the enzyme that could be largely prevented by addition of n^3PdAD^+. These results indicate that carbocyclic-analogs of NAD^+ will be useful in identifying the substrate binding site of NAD^+ glycohydrolase.

NAD^+glycohydrolases (NADases; EC 3.2.2.5) are a class of enzymes widely distributed in mammalian systems that are classically known for hydrolyzing $NAD(P)^+$ to adenosine diphosphate ribose (ADP-ribose) and nicotinamide [1]. These enzymes, which are nearly all membrane-bound proteins, are also characterized by their ability to catalyze transglycosidation reactions [1,2]. Calf spleen NADase, whose kinetic and catalytic mechanisms of action were the most extensively investigated [2-6], was purified to homogeneity [7]. Despite the fact that NADases from different sources have been studied for many decades, both from catalytic and cellular perspectives, their biochemical functions remain still poorly understood. Importantly, although some NADase activity was found associated with intracellular compartments such as mitochondria [8,9], NAD^+glycohydrolases are overwhelmingly ecto-enzymes in cells that express high levels of this enzyme activity [10,11].

Recently, related enzyme activities have been described which open new perspectives for NAD^+glycohydrolases. They are involved in the biosynthesis and catabolism of cyclic ADP-ribose (cADPR), a new metabolite, originally discovered in sea urchin eggs, which is thought to be an endogenous regulator of the Ca^{2+}-induced Ca^{2+}-release process mediated by the ryanodine receptors [12]. The first enzyme, i.e. ADP-ribosyl cyclase, was discovered in invertebrates and converts NAD^+ exclusively into the cyclic form of ADP-ribose [13,14]. In mammalian tissues no equivalent activity could be detected; however, a high sequence homology was found between this cyclase and CD38, a human lymphocyte cell surface antigen [15]. CD38 proved to be a multifunctional enzyme that, besides catalyzing the hydrolysis of NAD^+ into ADP-ribose, was also able to produce cADPR (less than 2-3% of reaction products) and to hydrolyze cADPR into ADP-ribose [16]. Similar catalytic activities were established independently for a canine spleen NAD^+glycohydrolase [17]. Recently we have shown that purified calf spleen NAD^+glycohydrolase was indeed also a multifunctional enzyme and have determined the molecular mechanism of cADPR formation [18].

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The molecular structure of the NAD^+ metabolizing enzymes is under intense investigation; e.g., crystal structure of *Aplysia* ADP-ribosyl cyclase is currently being solved [19] and the structure of NAD^+ bound to diphtheria toxin was recently published [20]. To identify the amino acid residues of the substrate binding domain of calf spleen NAD^+glycohydrolase, two non-cleavable photoactive analogs of NAD^+, 8-azidoadenosyl carba-NAD^+ (**3;** Fig. 1) and 8-azidoadenosyl pseudocarba-NAD^+ (**4**) have been tested for their ability to photoinactivate and photolabel the active site of purified calf spleen NAD^+glycohydrolase.

MATERIALS AND METHODS

Carba-NAD^+ and pseudocarba-NAD^+ and their corresponding photoactive 8-azidoadenosyl analogs were prepared as reported previously [21-23]. 3-Aminopyridine adenine dinucleotide (n^3PdAD^+) was obtained from NAD^+ by pig brain NADase catalyzed transglycosidation as described before [24], whereas β-NAD^+ and 1,N^6-etheno-NAD^+ were from Sigma. NAD^+ glycohydrolase was solubilized from calf spleen microsomes with a non-ionic detergent and purified to homogeneity according to our procedure described previously [7]; its specific activity was about 180 U/mg protein. The protein concentration was determined by the bicinchoninic acid (BCA) protein assay from Pierce using bovine serum albumin as standard.

[*2-Adenosyl-^3H]-8-azidoadenosyl carba-NAD^+ ([^3H]-**3**).* [2-^{3}H]-AMP (Amersham) was converted to [2-^{3}H]-8-N_3-AMP in two steps by modification of the procedure of Czarnecki et al. [25]. [2-^{3}H]-AMP (5 mCi, 0.078 mmol, 27 mg) was brominated and the [2-^{3}H]-8-Br-AMP purified by anion-exchange chromatography on a 2 $\times$ 10 cm column of Dowex AG 1X2 resin, 100-200 mesh, acetate form. The mononucleotide was applied in distilled water and the column developed using a linear gradient formed between 90 ml each of water and 4N acetic acid. 8-Br-AMP eluted at the end of the gradient. The displacement of the 8-bromo group to produce [2-^{3}H]-8-N_3-AMP was conducted as described [25] except that tetrabutylammonium azide was used and the product was purifies on a Dowex column as described above.

[2-^{3}H]-8-N_3-AMP was chemically coupled to 2′,3′-di-O-acetylcarbanicotinamide 5′-mono-phosphate [21] and the acetyl protecting groups removed to produce [^{3}H]-**3** according to the published procedure for the synthesis of unlabeled **3** [21,22]. The purity of the dinucleotide was verified using HPLC (RSIL AN, 4.6 $\times$ 250 mm developed with 250 mM KH_2PO_4, pH 3.5 at 1 ml/min) where a single peak was observed isographic with authentic **3.**

NAD^+glycohydrolase assay. The activity of NAD^+glycohydrolase was determined by a sensitive continuous fluorometric method [10], using 1,N^6-etheno-NAD^+ (50 μM) in the presence of 600 μM NAD^+ (saturating substrate conditions). The assay, run at 37°C, consisted in measuring the fluorescence emission at 410 nm (excitation 310 nm) of the reaction product in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1% (w/v) Emulphogene BC-720 (final volume: 2 ml). One unit of enzyme is the amount that consumes 1 μmol of NAD^+/min.

Inhibition of NAD^+glycohydrolase by carbocyclic analogs of NAD^+. The inhibition of NAD^+glycohydrolase was determined according to Lineweaver-Burk. Initial rates of 1,N^6-etheno-NAD^+ (14 - 800 μM) hydrolysis were determined fluorometrically, at 37°C, in a 10 mM potassium phosphate buffer (pH 7.4) containing 0.05% Emulphogene BC-720, in the presence of 15 milliunit of enzyme and **1** (0-80 μM), **2** (0-20 μM), **3** (0-40 μM) or **4** (0-10 μM). The assays with **3** and **4** were run in the dark. Kinetic parameters (V and K_{app}) were determined, at constant inhibitor concentrations, by fitting the Michaelis-Menten equation with a non-linear regression program. Inhibition constants were obtained from K_{app} vs. [I] secondary plots.

*Photoinactivation of calf spleen NAD^+glycohydrolase by 8-azidoadenosyl carba-NAD^+ (**3**) and 8-azidoadenosyl pseudocarba-NAD^+ (**4**).* For irradiation experiments, monochromatic light was obtained from a Xe/Hg lamp of 1000 Watts connected to a monochromator (Kratos). The light intensity was modulated by a diaphragm and monitored with a thermobattery (Kipp and Zohnen) coupled with a microvoltmeter. A solution of enzyme (about 0.56 Unit) in 10 mM potassium phosphate buffer (pH 7.4) containing 0.5 % CHAPS (final volume: 0.8 ml) was irradiated in the presence of 8-azidoadenosyl carba-NAD^+ and 8-azidoadenosyl pseudocarba-NAD^+ (at concentrations indicated in the text) at 254 nm (50 μV) and 10°C in a 1 cm pathlength quartz cell under gentle stirring. At times indicated, 40 μl aliquots were removed, and the residual NADase activity was determined. These operations were repeated in the presence of a protective ligand, 3-aminopyridine adenine dinucleotide (160 μM), added at time zero.

Analysis of the covalent photoaffinity labeling of calf spleen NAD^+glycohydrolase. About 50 μg of purified enzyme was irradiated under the conditions described above (final volume: 1ml) in the presence of 22.5 μM 2-[^{3}H]-8-azidoadenosyl carba-NAD^+ (1.5 10^6 dpm) for 20 min. For protection experiment, the same operation was repeated with addition of n^3PdAD (160 μM). After irradiation, the samples were concentrated (Amicon). Aliquots (about 3 μg of protein) were heated for 3 min in a boiling water bath in a 125 mM Tris,HCl buffer, pH 6.8, containing 2% SDS, 0.05% (w/v) 2-mercaptoethanol and analyzed by 12.5% SDS-PAGE according to Laemmli [26]. A mixture of molecular mass standards was also run (low molecular calibration kit from Pharmacia). Proteins were silver-stained according to the method of Morrissey [27]. For quantitative determination of radioactivity, the gel was cut into 2.1 mm slices and each slice was digested for 12 h at 80 °C with 200 μl hydrogen peroxide. Then, 200 μl 4 M urea/ 1% SDS and

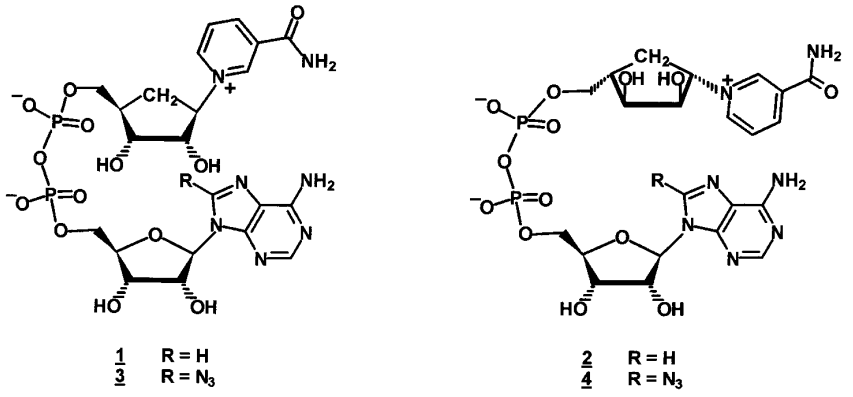

FIG. 1. Structures of carba-NAD$^+$ (**1**), pseudocarba-NAD$^+$ (**2**) and their photoreactive derivatives 8-azidoadenosyl carba-NAD$^+$ (**3**) and 8-azidoadenosyl pseudocarba-NAD$^+$ (**4**).

4 ml scintillation cocktail (Emulsifier-Safe, Packard) were added in each vial and the mixture was allowed to stand 8 h before counting.

RESULTS AND DISCUSSION

To efficiently label the active site of NAD$^+$glycohydrolase with a photoaffinity probe it is important that such a molecule is derived from a non-hydrolyzable analog of NAD$^+$, or at least from a very poor substrate, presenting a good affinity for the enzyme. Carbocyclic analogs of NAD$^+$, in which the nicotinamide-ribose ring oxygen has been replaced with a methylene group, should meet such requirements. We have first studied the inhibition of calf spleen NAD$^+$glycohydrolase by such molecules, then tested the photoinactivation of this enzyme by their photoactive derivatives.

Inhibition of calf spleen NAD$^+$glycohydrolase by carbocyclic analogs of NAD$^+$. The structures of carba-NAD$^+$ (**1**) and pseudocarba-NAD$^+$ (**2**) are shown on Fig. 1. In **1,** the D-ribotide ring of the substrate is replaced by [1*R*]-dihydroxycyclopentane methanol, the carbocyclic analog of D-ribose, and in **2** by the carbocyclic analog of L-ribose. Previous work had established that neither **1** nor **2** were substrates for closely related enzymes [22]. Addition of these analogs to calf spleen NAD$^+$glycohydrolase resulted in an inhibition of its activity; analysis of kinetic data (not shown) indicated a linear competitive type of inhibition mechanism with Ki = 20 μM and 7 μM respectively for **1** and **2.** The surprising result that the pseudocarba-NAD$^+$ analog, despite its configuration which is related to an unnatural diastereoisomer of NAD$^+$, is an excellent inhibitor was observed before with purified *Bungarus fasciatus* venom enzyme and crude mammalian NADases [23]. However, is contrast to these earlier works where carba-NAD$^+$ was found to be a weak inhibitor [23], this compound has also a good affinity for the purified calf spleen enzyme.

The same type of experiments were performed with the 8-azidoadenosyl derivatives of carba-NAD$^+$ (**3;** Fig. 1) and pseudocarba-NAD$^+$ (**4**) and gave respectively Ki values of 25 μM and 9 μM (Fig. 2); the similitude of Ki with the parent compounds **1** and **2** indicates no important changes in binding consecutive to the introduction of the azido group into the adenine moiety.

Photodecomposition of the carbocyclic analogs of NAD$^+$. To determine the optimal irradiation conditions for the photoinactivation of calf spleen NAD$^+$glycohydrolase we have first studied the photodecomposition of **3** and **4** under our experimental conditions. From the photodependent spectral changes in absorbance upon irradiation at 254 nm, half times of 12 and 10 min were determined respectively for **3** and **4** (not shown).

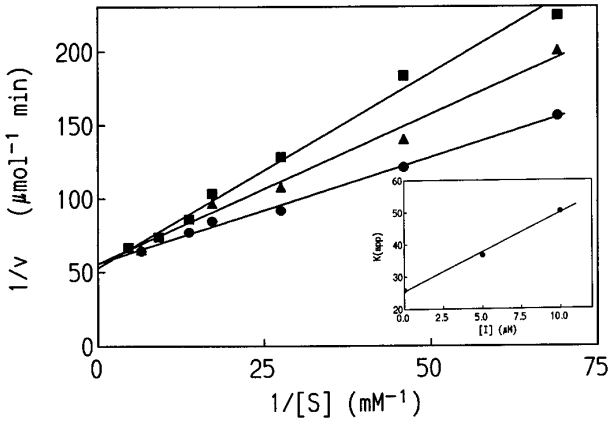

FIG. 2. Inhibition of bovine spleen NAD^+glycohydrolase by 8-azidoadenosyl pseudocarbaNAD^+ (**4**). Initial rates of 1,N^6-etheno-NAD^+ hydrolysis were determined fluorometrically at 37°C, in 10 mM potassium phosphate buffer (pH 7.4) containing 0.05% (w/v) Emulphogene (final volume: 2 ml), in the presence of 15 milliunits enzyme. Lineweaver-Burk representation of the inhibition at 0 (- ● -), 5 (- ▲ -) and 10 μM (- ■ -) of **4.** *Inset:* Replot of the apparent Km versus inhibitor concentrations.

Photodependent inhibition of NAD^+glycohydrolase by 8-azidoadenosyl carba and 8-azidoadenosyl pseudocarba-NAD^+. Preliminary experiments were performed to find irradiation conditions allowing a good compromise between the photodecomposition of the photoreactive analogs and the enzyme resistance. Under our experimental conditions, i.e. irradiation at 254 nm at 10°C, photodependent inhibition of calf spleen NAD^+glycohydrolase was observed in the presence of **3** and **4** used at concentrations in the range of their Ki. Although the enzyme itself is somewhat sensitive to irradiation, efficient photoinactivation was obtained especially with the 8-azidoadenosyl pseudocarba-NAD^+ (**4**) (Fig. 3); on longer irradiation times the effect plateaus at about 30% residual activity. The photodependent inhibition of NAD^+glycohydrolase by these compounds could be completely prevented in the presence of n^3PdAD^+ (Fig. 3), a known potent competitive inhibitor of the enzyme (Ki = 5 μM)[24], suggesting that compounds **3** and **4** photoinactivate the enzyme by binding to its active site. Interestingly, n^3PdAD^+ not only protects the enzyme from photoinhibition by **3** and **4,** but it also abolishes the NADase

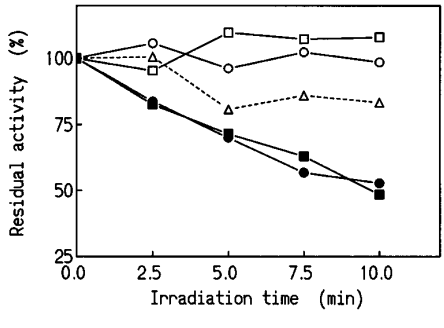

FIG. 3. Photoinactivation of bovine spleen NAD^+glycohydrolase. The enzyme (0.56 U, 4 μg) was irradiated at 254 nm and 10°C with 10 μM **3** (- ● -) or **4** (- ■ -) in the absence or presence (open symbols) of 160 μM n^3PdAD^+. As a control, the irradiation was repeated in the absence of inhibitor (- △ -). At times indicated, the residual enzyme activity was measured fluorometrically on aliquots (40 μl), under saturating conditions, as described under Materials and Methods.

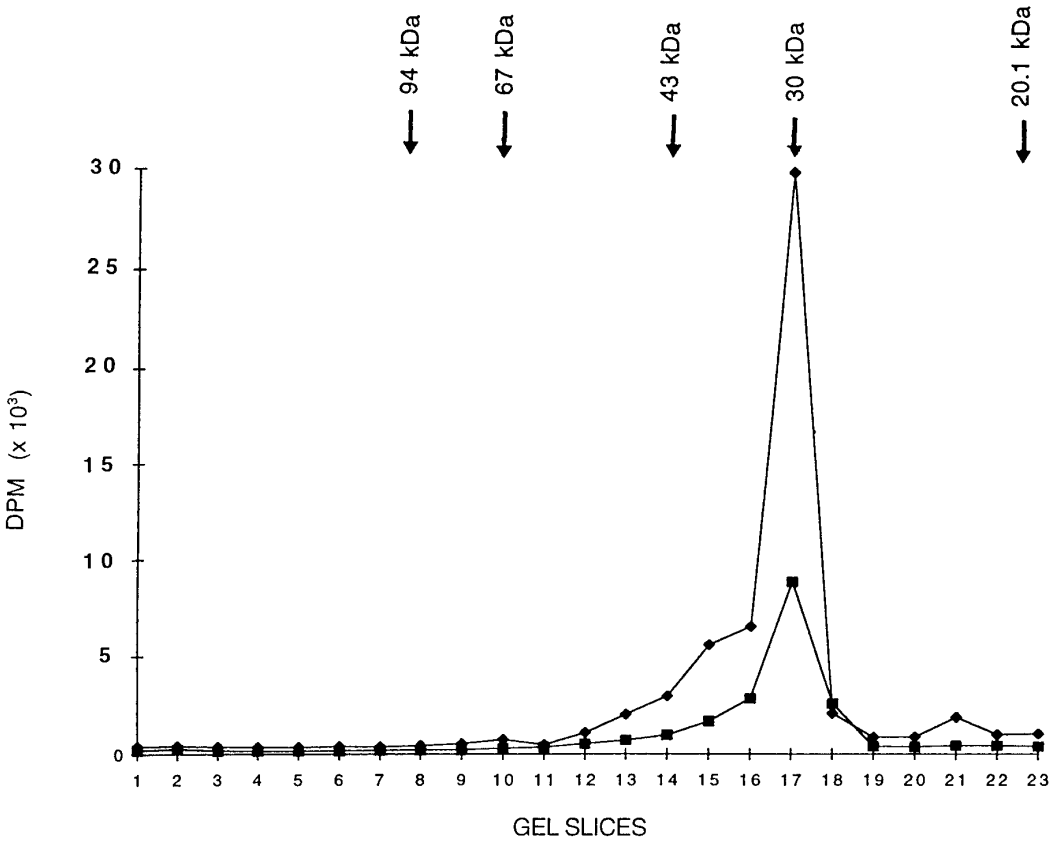

FIG. 4. Electrophoretic analysis of labeled bovine NAD^+glycohydrolase. Bovine spleen NADase (50 μg) was irradiated at 254 nm in the presence of 22.5 μM 2-^{3}H-8-azidoadenosyl carba-NAD^+ (**3**) in the absence (- ◆ -) or in the presence of 160 μM 3-aminopyridine adenine dinucleotide (- ■ -). Samples were analyzed on SDS-PAGE as described under Materials and Methods.

photoinactivation observed in the absence of the photolabels. Occupation of the active site by a ligand might mask a residue, e.g. tryptophan, sensitive to irradiation at 254 nm, the wavelength used in our experiments.

Labeling of NAD^+glycohydrolase with radioactive 8-azidoadenosylcarba NAD^+. We studied the incorporation of [^{3}H]-labeled **3** into purified bovine spleen NAD^+glycohydrolase. After irradiation, the samples were analyzed by gel electrophoresis; under denaturing conditions, and the radioactivity could be attributed exclusively to stable irreversible labeling; under our experimental conditions, 45% of the enzyme was labeled. Fig. 4 gives a SDS-PAGE profile of labeled NAD^+glycohydrolase and indicates that the radioactivity incorporated into the enzyme (MW 30 kDa) is much decreased when the irradiation was carried out in the presence of protecting n^3PdAD^+. These results establish the high yield and specificity of photolabeling of NAD^+glycohydrolase with the carbocyclic analogs of NAD^+.

CONCLUSION

8-Azidoadenosyl carba-NAD^+ (**3**) and 8-azidoadenosyl pseudocarba-NAD^+ (**4**), two non-hydrolyzable photoreactive analogs of NAD^+ were found to be potent competitive inhibitors of purified calf spleen NAD^+glycohydrolase. The efficient photoinactivation of the enzyme by

these compounds, which can be abolished in the presence of a non-photoactive competitive inhibitor, will render radiolabeled **3** and **4** excellent tools for probing the molecular structure of its active site.

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