Enzymatic Synthesis of NAD^+ with the Specific Incorporation of Atomic Labels†

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Abstract: An enzymatic synthesis is described for the production of NAD^+ labeled with a radioactive or stable isotope at any desired position in the AMP or NMN^+ portions of the molecule. In the first step, ten enzyme-catalyzed reactions are coupled for the synthesis of nicotinic acid adenine dinucleotide ($NaAD^+$) from glucose, nicotinic acid, and ATP. NAD^+ is formed from $NaAD^+$ and glutamine in the second step. Oxidized nicotinamide adenine dinucleotide was synthesized with ^3H, ^{14}C, or ^{15}N label specifically incorporated in the ribose or nicotinamide of the NMN^+ portion of NAD^+ as [H_N1'-^3H]NAD^+, [H_N2'-^3H]NAD^+, [H_N4'-^3H]NAD^+, [H_N5'-^3H]NAD^+, [C_N1'-^{14}C]NAD^+, [C_N5'-^{14}C]NAD^+, [N_N1-^{15}N, C_N1'-^{14}C]NAD^+, and [N_N1-^{15}N, C_N5'-^{14}C]NAD^+. Nuclear magnetic resonance spectroscopy of [H_N2'-^2H]NAD^+ as well as enzymatic degradation were used to verify the position of labels. Appropriately labeled glucose, ribose 5-phosphate, or nicotinic acid were the starting materials and were converted to NAD^+ using enzymes from the pentose pathway and the pathway for NAD^+ *de novo* synthesis. Yields of purified NAD^+ to 96% were obtained from starting glucose. The labeled NAD^+ is catalytically competent and is chromatographically and spectrophotometrically indistinguishable from authentic NAD^+. By using specifically labeled ATP as a precursor (Parkin, D. W.; Schramm, V. L. *Biochemistry* **1987**, *26*, 913–920), the method is readily adaptable for the synthesis of NAD^+ with single or multiple atomic labels at various positions in the AMP portion of the molecule. NAD^+ was synthesized from [8-^{14}C]ATP to give [C_A8-^{14}C]NAD^+ as an example. Together these methods provide a general scheme for the efficient synthesis of NAD^+ of high purity with ^3H, ^{14}C, or other labels at any nonexchangeable position of the NMN^+ or AMP portions of the NAD^+ molecule.

Introduction

ADP-ribosylation and related reactions are important in a range of biological modifications including the action of bacterial toxins,[1,2] DNA repair mechanisms,[3–5] regulation of calcium ion concentration[6,7] and regulation of chromatin structure.[8,9] Kinetic isotope effects using appropriately labeled NAD^+ molecules could provide valuable tools for the study of these reactions. Industrial scale syntheses of NAD^+ have been described by a combination of chemical and enzymatic methods, as has a synthesis of [ribose-(NMN)-^{14}C]NAD^+ from [U-^{14}C]glucose using cell extracts of *Lactobacillus fructosus* and purified enzymes.[10–12] Based on previously reported methods for the enzymatic synthesis of purine nucleotides and nucleosides,[13–15] an enzymatic synthesis for NAD^+ was designed using the 11 enzymes required to convert glucose, nicotinic acid, and ATP into NAD^+ with the catalytic cycling of cofactors. The purpose of this study was to develop a general enzymatic method for the specific, high-efficiency incorporation of specific labels into NAD^+ to be used for the measurement of kinetic isotope effects.

A procedure was developed for the enzymatic synthesis of NAD^+ with the incorporation of specific ^3H and ^{14}C labels in the ribose ring bound to nicotinamide, ^{15}N label in nicotinamide, and any desired label in the AMP portion of NAD^+. This procedure uses specifically labeled glucose, ribose 5-phosphate, nicotinic acid, and ATP as starting materials. Enzymatic synthesis of NAD^+ is stereospecific, proceeds with high yields, and eliminates the byproducts generated during chemical synthesis. Most of the enzymes required for the synthetic procedure are commercially available or can be readily obtained by published methods or methods described here. Labeled NAD^+ produced by these methods can be enzymatically converted to NADH, $NADP^+$, and NADPH. Specifically labeled NAD^+'s and related dinucleotides should prove useful for kinetic and radiolabel tracer studies of a variety of enzymes, including NAD^+ glycohydrolases, ADP-ribosyltransferases, NAD^+ cyclase, and enzymes of NAD^+-dependent oligonucleotide repair, such as poly(ADP-ribose) polymerase and poly(ADP-ribose) glycohydrolase.

Materials and Methods

Materials. [2-^3H]Glucose, [5-^3H]glucose, and [6-^3H]glucose were purchased from Amersham and were supplied with specific radioactivity of 9–36 Ci/mmol. [6-^{14}C]Glucose was from the same source with specific

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radioactivity of 54 mCi/mmol. [2-^{14}C]Glucose was purchased from American Radiolabeled Chemicals, Inc. and was supplied with specific radioactivity of 55 mCi/mmol. [8-^{14}C]ATP, with specific radioactivity of 54 mCi/mmol, was purchased from ICN Biomedicals, Inc. [1-^{15}N]-nicotinamide (99% label) was the generous gift of Dr. Norman J. Oppenheimer, University of California School of Pharmacy, San Francisco.[16] [2-^{2}H]Ribose 5-phosphate and [2-^{3}H]ribose 5-phosphate were produced by phosphoriboisomerase-catalyzed exchange from $^{2}H_2O$ or $^{3}H_2O$ solvent, respectively. The Bio-Rad $^{2}H_2O$ was 100.0 atom % grade, and the specific radioactivity of the $^{3}H_2O$ was 9 Ci/mmol. HPLC reverse phase C_{18} μBondapak was from Waters Associates. Alcohol dehydrogenase (1.1.1.1), hexokinase (2.7.1.1), and glucose 6-phosphate dehydrogenase (1.1.1.49) type VII from Bakers yeast, glucose 6-phosphate dehydrogenase (1.1.1.49) type XXIV from *Leuconostoc mesenteroides*, pyruvate kinase (2.7.1.40) type II and myokinase (2.7.4.3) from rabbit muscle, 6-phosphogluconate dehydrogenase (1.1.1.44) type V and phosphoriboisomerase (5.3.1.6) from *Torula* yeast, glutamate dehydrogenase (1.4.1.3) type I from bovine liver, NAD$^+$ glycohydrolase (3.2.2.5) from *Neurospora crassa* and nucleotide pyrophosphatase (3.6.1.9) from *Crotalus adamanteus* venom were purchased from Sigma. NAD$^+$ pyrophosphorylase (2.7.7.1) from hog liver was purchased from Boehringer Mannheim. NAD$^+$ synthetase (6.3.5.1) from Bakers' yeast was isolated as described below. NAD$^+$ synthetase from *E. coli* recently became available from Sigma and was used as supplied. 5-Phosphoribosyl-1-pyrophosphate synthetase (2.7.6.1) was a generous gift of Dr. Robert Switzer, University of Illinois, Urbana and is also available from Sigma. Nicotinate phosphoribosyltransferase (2.4.2.11) was the generous gift of Dr. Charles Grubmeyer, Temple University School of Medicine, Philadelphia, and can be obtained from an overexpression construct using the gene from *Salmonella*.[17,18]

Preparation of NAD$^+$ Synthetase from Bakers' Yeast. The partial purification of NAD$^+$ synthetase was modified from the method of Yu and Dietrich.[19] Protein was measured by a dye-binding method[20] using reagents purchased from Bio-Rad. Purification steps were at 4 °C, and buffer solutions contained 1 mM dithiothreitol and 1 mM EDTA. Bakers' yeast blocks purchased locally (2.7 kg) were suspended in 50 mM Tris, pH 7.4. The cells were disrupted in the presence of 7.5 μM phenylmethylsulfonylfluoride, 0.2 μM leupeptin, and 1.5 μg/mL pepstatin using glass beads in a Dyno-Mill apparatus from Impandex, Inc. The broken cell suspension, approximately 5 L, was centrifuged at 14 000 × g for 40 min. The pellet was resuspended in a minimal volume of 50 mM Tris, pH 7.4, without the protease inhibitors, and centrifuged at 40 000 × g for 45 min. The resulting supernatant was brought to 35% saturation with solid ammonium sulfate and allowed to equilibrate for 1 h with constant stirring. The precipitate was collected by centrifugation at 27 000 × g for 20 min and was resuspended in a minimal volume of 50 mM Tris, pH 7.4. The suspension was dialyzed against five changes of 60 volumes of 40 mM Tris, pH 7.2, 25 mM KCl. The turbid dialysate was diluted 1:1 in dialysis buffer and applied to a DEAE-cellulose column (2.5 × 58 cm), equilibrated with 40 mM Tris, pH 7.2, 40 mM KCl. The column was developed with a linear gradient from 40 to 260 mM KCl, and a single peak of enzymatic activity was obtained. Active fractions were pooled and concentrated to 18 mg/mL protein by collodion bag ultrafiltration (25 or 75 kDa M_r retention). The preparation was frozen in dry ice/ethanol and stored at −70 °C. The results of a typical partial purification of NAD$^+$ synthetase are listed in Table 1.

The assay solution for NAD$^+$ synthetase consisted of 50 mM Tris, pH 8.0, 2 mM ATP, 56 mM KCl, 5 mM $MgCl_2$, 20 mM glutamine, 0.2 mg/mL bovine serum albumin, 1 mM NaAD$^+$, 10 mM glucose 6-phosphate, and 9 U of glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* in a volume of 1 mL at 37 °C. Reactions were started by the addition of a sample from the NAD$^+$ synthetase preparation, and the change in A_{340} was used to estimate enzymatic activity. The NAD$^+$ synthetase activity was confirmed by HPLC which demonstrated the conversion of NaAD$^+$ to NAD$^+$.

Synthesis of [2-^{3}H]Ribose 5-Phosphate. Ribose 5-phosphate (20 mM, pH 7.0) was equilibrated with 100 units/mL phosphoriboisomerase in otherwise unbuffered H_2O.[21] A sample of the equilibrium mixture containing 3 μmol pentose phosphate was freeze-dried and reconstituted with 0.1 mL of H_2O containing 50 Ci $^{3}H_2O$. After 90 min at room temperature, the reaction was terminated by heating the sealed reaction vessel to 105 °C for 5 min. Following removal of excess $^{3}H_2O$, the proton-decoupled tritium-NMR gave the expected ratio of ^{3}H peaks for α-D-[2-^{3}H]ribose 5-phosphate, β-D-[2-^{3}H]ribose 5-phosphate, and D-[1-^{3}H]ribulose 5-phosphate as well as substantial tritium in high molecular weight material. The tritium labeling experiment was accomplished at the National Tritium Labeling Facility, Lawrence Berkeley Laboratory, under the skilled guidance of Hiromi Morimoto. [2-^{3}H]Ribose 5-phosphate was partially purified by G-10 Sephadex chromatography with 20 mM acetic acid as eluent, prior to use in NAD$^+$ synthesis.

Synthesis of [2-^{2}H]ribose 5-phosphate, for the purpose of NMR characterization, used an equivalent freeze-dried equilibrium mixture of phosphoriboisomerase and pentose phosphate as for ^{3}H-labeling. A sample containing 20 μmol pentose phosphate was reconstituted in 1 mL of 100.0 atom % grade $^{2}H_2O$ and treated exactly as described above. Following termination, the reaction was freeze-dried and used directly in NAD$^+$ synthesis.

Conversion of [1-^{15}N]Nicotinamide to [1-^{15}N]Nicotinic Acid. The substrate specificity of nicotinate phosphoribosyltransferase required conversion of [1-^{15}N]nicotinamide (99% label) to [1-^{15}N]nicotinic acid before use in the synthesis of [N_N1-^{15}N]-labeled NAD$^+$. [1-^{15}N]-Nicotinamide (5 mg) was refluxed for 2–3 h in 1 N HCl (0.5 mL), resulting in quantitative conversion of nicotinamide to nicotinic acid. The reaction mixture containing [1-^{15}N]nicotinic acid was dried under vacuum and resolubilized in distilled water.

General Synthesis of ^{3}H- and ^{14}C-Labeled NAD$^+$ from Labeled Glucose. Labeled NAD$^+$ was synthesized in a two-step process. In the first step labeled glucose or ribose 5-phosphate was converted to labeled NaAD$^+$ which was purified. In the second step, NaAD$^+$ was converted to NAD$^+$ using NAD$^+$ synthetase. NAD$^+$ synthetase activity from both Bakers' yeast and *E. coli* was inhibited by components of the first reaction mixture, thus requiring purification of NaAD$^+$ prior to NAD$^+$ formation. The *E. coli* NAD$^+$ synthetase is reported to prefer ammonia, rather than glutamine, as the nitrogen donor. The enzyme rapidly lost activity when stored in 50% glycerol at −20 °C and was inactive after two months. The scale of the synthesis was from 1 μmol labeled precursor or limiting reagent, resulting in a recovery of 0.5–0.9 μmol purified, labeled NAD$^+$. The reaction can be run at any scale, thus a 1 L reaction would be expected to provide 0.5–0.9 mmol of NAD$^+$.

Table 1. Partial Purification of NAD$^+$ Synthetase from Bakers' Yeast

step	total protein, mg	total activity,[a] units	specific activity, units/mg	yield, %
cell extract	31200	47	0.0015	100
$(NH_4)_2SO_4$ fractionation	3600	79.2	0.022	168
DEAE-cellulose column	84	29.4	0.346	62
ultrafiltration concentrate	82	18.5	0.225	39

[a] One unit catalyzes the formation of 1 μmol of NADH per min in the coupled assay procedure described in the methods.

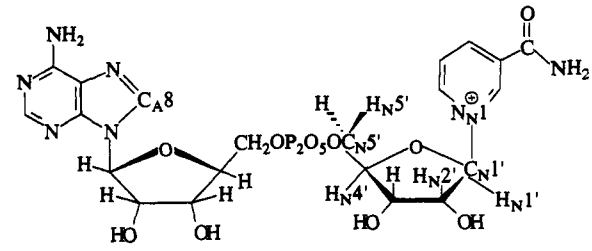

Figure 1. Structure of NAD$^+$ indicating the numbering of atoms in the molecule where radioactive or heavy atom isotopes were incorporated. Subscripts N and A indicate the NMN$^+$ and AMP portions of the molecule, respectively. Primed numbers indicate atomic locations in the ribosyl portions of the molecule.

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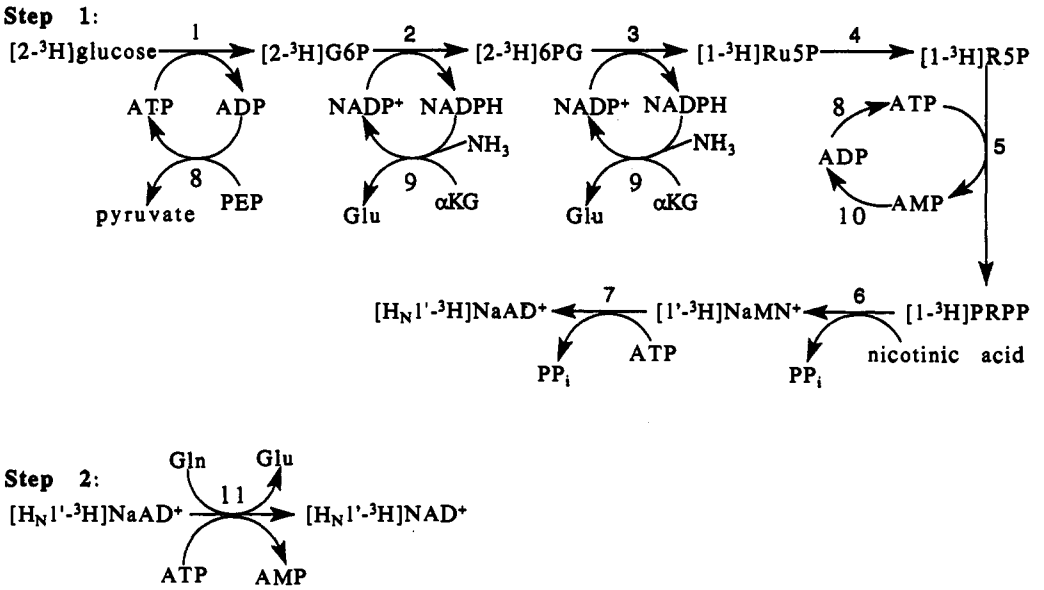

Figure 2. Enzymatic synthesis of NAD+ from labeled glucose, showing synthesis of [H_N1'-^{3}H]NAD+ as an example. Enzymes are (1) hexokinase, (2) glucose 6-phosphate dehydrogenase, (3) 6-phosphogluconate dehydrogenase, (4) phosphoriboisomerase, (5) 5-phosphoribosyl-1-pyrophosphate synthetase, (6) nicotinate phosphoribosyltransferase, (7) NAD+ pyrophosphorylase, (8) pyruvate kinase, (9) glutamate dehydrogenase, (10) adenylate kinase, and (11) NAD+ synthetase. Enzymes 1–10 and all reagents for the conversion of glucose to NaAD+ are incubated in a single reaction mixture in step 1. The resulting NaAD+ is purified and converted to NAD+ in a second reaction indicated as step 2. Abbreviations in the figure are G6P, glucose 6-phosphate; 6PG, 6-phosphogluconic acid; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; NaMN+, nicotinic acid mononucleotide; NaAD+, nicotinic acid adenine dinucleotide; PEP, phosphoenolpyruvate; and αKG, α-ketoglutarate.

(A) Synthesis of Labeled NaAD+. NaAD+ was synthesized in a 1 mL reaction mixture consisting of 50 mM potassium phosphate, pH 7.5, 3 mM $MgCl_2$, 50 mM KCl, 5 mM dithiothreitol, 2 mM ATP, 10 mM phosphoenolpyruvate, 10 mM α-ketoglutarate, 0.1 mM NADP+, 2 mM nicotinic acid (pH 6.2), and 25–100 μCi of either ^{3}H- or ^{14}C-glucose. [2-^{3}H]-, [5-^{3}H]-, [6-^{3}H]-, [2-^{14}C]-, and [6-^{14}C]glucose were used as the sources of label for [H_N1'-^{3}H]-, [H_N4'-^{3}H]-, [H_N5'-^{3}H]-, [C_N1'-^{14}C]-, and [C_N5'-^{14}C]NAD+, respectively (see Figure 1 for atom assignments). Unlabeled glucose was added to give a total glucose concentration of 1 mM. All enzymes, with the exception of nicotinate phosphoribosyltransferase, were diluted into 50 mM potassium phosphate, pH 7.5, and used as follows: 0.1 U/mL hexokinase, 2 U/mL pyruvate kinase, 0.1 U/mL glucose 6-phosphate dehydrogenase, 0.5 U/mL glutamate dehydrogenase, 0.1 U/mL 6-phosphogluconate dehydrogenase, 6 U/mL phosphoriboisomerase, 0.5 U/mL myokinase, 0.25 U/mL NAD+ pyrophosphorylase, and 0.2 U/mL 5-phosphoribosyl-1-pyrophosphate synthetase. Nicotinate phosphoribosyltransferase, supplied as an ammonium sulfate precipitate, was collected by centrifugation, resuspended in 25 mM potassium phosphate, pH 7.5, and stored at −70 °C. This preparation was used in a quantity of 0.02 U/mL. Samples of labeled glucose were dried under vacuum before use. All enzymes, except hexokinase, were combined, followed by addition of other reagents in the order listed. Hexokinase was added to start the reaction. The reaction mixture was incubated for 4 h at 37 °C. Synthesis of NaAD+ was monitored by HPLC at 260 nm using a reverse phase C_{18} μBondapak column (7.8 × 300 mm) in 0.1 M ammonium acetate, pH 5.0.

Reactions were terminated by placing the plastic reaction vial in a heating block (120 °C) for 1.5 min. The solution was clarified by centrifugation at 15 000 × g for 5 min. Partial purification of the NaAD+ was accomplished by HPLC under the same conditions used to monitor NaAD+ production, as described above. The supernatant (1 mL) was injected in two aliquots through a 0.5 mL injection loop at a flow rate of 2 mL/min. For each injection, the NaAD+ peak was collected directly into a lyophilization flask. The total product was frozen and lyophilized to dryness. AMP coeluted with NaAD+ in this chromatography system but was present at low concentrations due to the ATP-regenerating system provided by pyruvate kinase and myokinase.

(B) Synthesis of Labeled NAD+ from Labeled NaAD+. The isolated NaAD+ was resuspended in a 0.5 mL reaction mixture to give a final concentration of approximately 2 mM. The reaction mixture included 50 mM potassium phosphate, pH 7.5, 50 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 4 mM ATP, 20 mM glutamine, and 0.2 U/mL yeast NAD+ synthetase. The solution was incubated for 2 h at 37 °C.

(C) Purification, Storage, and Stability of Labeled NAD+. The labeled NAD+ was purified through HPLC by direct injection of the entire NAD+ synthetase reaction mixture (0.5 mL) under the same conditions used for isolation of NaAD+. The NAD+, which had a longer retention time than all other reaction components, was collected directly into a lyophilization flask, shell-frozen, and lyophilized to dryness. The purified NAD+ was resuspended in 1 mL of 50% ethanol. The final preparation was stored at −70 °C. After 12 months of storage under these conditions, HPLC analysis of [H_N1'-^{3}H]- and [H_N5'-^{14}C]NAD+ indicated that greater than 99% of applied radioactivity eluted with NAD+ in both cases. The stability of the labeled NAD+'s is comparable to that observed for unlabeled, salt-free NAD+ stored dry at −15 °C.[22]

Synthesis of [H_N2'-^{3}H]NAD+ from Labeled Ribose 5-Phosphate. Synthesis of [H_N2'-^{3}H]NAD+ from [2-^{3}H]ribose 5-phosphate was accomplished in a 1 mL reaction mixture containing 50 mM potassium phosphate, pH 7.5, 3 mM $MgCl_2$, 50 mM KCl, 5 mM dithiothreitol, 2 mM ATP, 10 mM phosphoenolpyruvate, 2 mM nicotinic acid (pH 6.2), 1 mM ribose 5-phosphate, and approximately 50 μCi of partially purified [2-^{3}H]ribose 5-phosphate. The enzyme mixture was as described above except for the omission of hexokinase, glucose 6-phosphate dehydrogenase, glutamate dehydrogenase, 6-phosphogluconate dehydrogenase, and phosphoriboisomerase. Purification of the NaAD+ and its conversion to NAD+ were as described above.

[H_N2'-^{2}H]NAD+ was synthesized as described for [H_N2'-^{3}H]NAD+ except that 20 μmol of carrier-free [2-^{2}H]pentose phosphate was used as the starting reagent. The scale of the synthesis was increased 3-fold to obtain a quantity sufficient for NMR spectroscopic analysis.

Synthesis of [N_N1-^{15}N, C_N1'-^{14}C]NAD+, [N_N1-^{15}N, C_N5'-^{14}C]NAD+, and [C_A8-^{14}C]NAD+. [N_N1-^{15}N, C_N1'-^{14}C]-, [N_N1-^{15}N, C_N5'-^{14}C]-, and [C_A8-^{14}C]NAD+ were also synthesized using modifications of the general synthetic scheme described above. [N_N1-^{15}N, C_N1'-^{14}C]NAD+ and [N_N1-^{15}N, C_N5'-^{14}C]NAD+ were synthesized as described for [C_N1'-^{14}C]- and [C_N5'-^{14}C]NAD+, respectively, except for the use of carrier-free [1-^{15}N]nicotinic acid (99% label). [C_A8-^{14}C]NAD+ was synthesized using unlabeled nicotinic acid mononucleotide (NaMN+) as the starting point in the synthetic pathway. The reaction mixture for incorporation of labeled ATP included 50 mM potassium phosphate, pH 7.5, 3 mM $MgCl_2$, 1 mM NaMN+, 1 mM ATP, and 25 μCi [8-^{14}C]ATP in a volume of 1 mL. The reaction was initiated with 0.25 U/mL NAD+ pyrophosphorylase and incubated at 37 °C for 30 min. The NaAD+ was purified and converted to NAD+ as described above.

Spectral Properties of Labeled NAD+. Labeled NAD+, prepared by the methods described above, was assayed by comparing the spectrophotometric properties of authentic NAD+ with a mixture of [H_N1'-^{3}H]- and [C_N5'-^{14}C]NAD+, before and after treatment with alcohol dehy-

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drogenase. Authentic and labeled NAD^+ solutions were prepared at matched concentrations in 50 mM HEPES, pH 8.0. Ethanol was added to both NAD^+ solutions to a final concentration of 10 mM, followed by addition of 0.1 U alcohol dehydrogenase. After 15 min for equilibration at 30 °C, the spectrum of each reaction mixture was again recorded for comparison of the NAD^+/NADH spectra and equilibrium.

Enzymatic Degradation of Labeled NAD^+. NAD^+ glycohydrolase and nucleotide pyrophosphatase were used to degrade labeled NAD^+ to ADP-ribose and nicotinamide and to AMP and NMN^+, respectively. For each reaction, a mixture of [H_N1'-^{3}H]- and [C_N5'-^{14}C]NAD^+ (2×10^5 cpm total) was dried under vacuum. The NAD^+ glycohydrolase reaction mixture consisted of 50 mM potassium phosphate, pH 7.0, 1 mM carrier NAD^+ and 0.2 U enzyme. The nucleotide pyrophosphatase reaction mixture consisted of 50 mM potassium phosphate, pH 7.0, 3 mM $MgCl_2$, 1 mM carrier NAD^+, and 1 U enzyme. After the reactions had gone to completion at 37 °C, the mixtures were analyzed by reverse phase HPLC eluted with 50 mM ammonium acetate, pH 5.0. Fractions were collected from HPLC injections and counted in a TM Analytic scintillation counter. The position of radiolabel was determined by the coelution of radioactivity with standards of ADP-ribose, NMN^+, and AMP.

NMR Spectroscopy of Labeled NAD^+. 500-MHz ^{1}H NMR spectra were recorded for a commercial preparation of NAD^+ (Sigma, 9 mM) and for [H_N2'-^{2}H]NAD^+ (5 mM) using a Varian 500-MHz spectrometer. Samples were in ^2H_2O solvent containing 50 mM potassium phosphate, pD 7.4. Chemical shifts were measured relative to the methyl resonance of 3-(trimethylsilyl)tetradeuterio sodium propionate. A line broadening of 1.5 Hz was applied, and 64 transients were accumulated, allowing measurement of peak heights of proton signals. Peaks were assigned based on published spectra.[23] The position of label incorporation was determined based on comparison of the H_N1' signal for [H_N2'-^{2}H]NAD^+ with that for authentic NAD^+.

Results

Synthesis of Labeled NAD^+. In all NAD^+ syntheses with label incorporated from sugars, $NaAD^+$ was produced first at yields up to 98% through the series of reactions shown in step 1 of Figure 2. Conversion of $NaAD^+$ to NAD^+ was also accomplished in yields as high as 98% using the NAD^+ synthetase reaction, shown in step 2 of Figure 2. Incorporation of radioactivity into $NaAD^+$ and NAD^+ was followed by HPLC (Figure 3). The overall yields of purified, labeled NAD^+ were in the range of 54–96% from starting sugar, or $NaMN^+$, for all NAD^+'s synthesized. The yields for synthesis of [H_N1'-^{3}H]NAD^+, [H_N2'-^{3}H]NAD^+, [H_N4'-^{3}H]NAD^+, [H_N5'-^{3}H]NAD^+, [C_N1'-^{14}C]-NAD^+, [C_N5'-^{14}C]NAD^+, [C_A8-^{14}C]NAD^+, [N_N1-^{15}N, C_N1'-^{14}C]NAD^+, and [N_N1-^{15}N, C_N5'-^{14}C]NAD^+ are summarized in Table 2.

Spectrophotometric and Catalytic Characterization of Labeled NAD^+. The spectrophotometric properties of labeled, enzymatically synthesized NAD^+ are indistinguishable from those of authentic NAD^+ (Figure 4). The catalytic competence of labeled NAD^+ was determined by comparing labeled and authentic NAD^+'s in the alcohol dehydrogenase reaction. An identical NAD^+/NADH equilibrium was obtained with unlabeled NAD^+ and a mixture of [H_N1'-^{3}H]- and [C_N5'-^{14}C]NAD^+. This experiment establishes that the labeled NAD^+'s are catalytically competent and contain no unreactive, UV-absorbing species since unreactive material would cause an apparent change in the equilibrium position relative to unlabeled NAD^+.

Enzymatic Degradation of Labeled NAD^+. Enzymatic degradation of labeled NAD^+ allowed the position of radiolabel to be located within a specific ribosyl group. Treatment of the labeled NAD^+ with nucleotide pyrophosphatase followed by HPLC demonstrated that all of the label from both [H_N1'-^{3}H]NAD^+ and [C_N5'-^{14}C]NAD^+ eluted with the NMN^+ portion of the molecule (Figure 5). Treatment with NAD^+ glycohydrolase resulted in elution of >85% of radioactivity with ADP-ribose

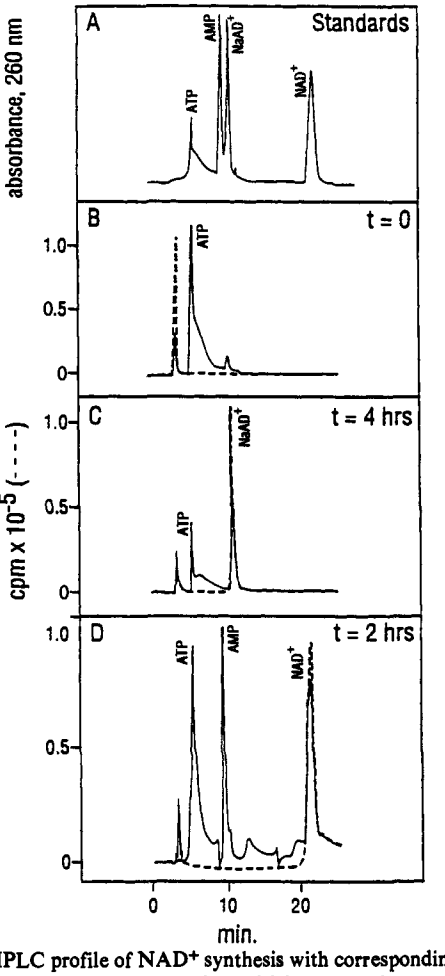

Figure 3. HPLC profile of NAD^+ synthesis with corresponding elution profile of radioactivity. For comparison with lower panels, panel A shows elution times of all UV-absorbing substrates and products of the synthesis. Panel B shows the elution profile of the $NaAD^+$ synthesis reaction mixture, with starting label as [6-^{14}C]glucose, before the reaction was initiated by the addition of hexokinase. Panel C shows the elution profile of the $NaAD^+$ synthesis reaction mixture at the time the reaction was terminated. The decrease in the ATP peak is apparent as is the increase in the $NaAD^+$ peak. For this experiment, the initial concentrations of ATP and glucose were 2 and 1 mM, while the final concentrations were approximately 1 and 0.1 mM, respectively, based on the absorbance of peaks detected by HPLC and the radioactivity elution profile. Panel D shows the production of NAD^+ by NAD^+ synthetase. The $NaAD^+$, used at an initial concentration of approximately 2 mM, is essentially gone, and the corresponding conversion of ATP to AMP is apparent. For this experiment, the initial concentration of ATP was 4 mM, while the final concentration was approximately 2 mM, based on the absorbance of peaks detected by HPLC.

(Figure 5). The remaining radioactivity showed no ultraviolet absorbance and eluted at the same position as ribose 5-phosphate. The commercial preparation of NAD^+ glycohydrolase contains a weak pyrophosphatase activity which causes the hydrolysis of ADP-ribose to AMP and ribose 5-phosphate. A chromatographic peak was observed at the position expected for AMP and was obtained for both the labeled and authentic NAD^+. Control experiments involving chromatography of labeled NAD^+ without enzymatic treatment did not produce analogous peaks of radioactivity. Thus, all detectable label in [H_N1'-^{3}H]NAD^+ and [C_N5'-^{14}C]NAD^+ is specifically incorporated into the ribose of the NMN^+ portion of NAD^+.

NMR Spectroscopy of Labeled NAD^+. The 500-MHz ^{1}H NMR spectrum of [H_N2'-^{2}H]NAD^+ unambiguously demonstrated the specific incorporation of label from [2-^{2}H]ribose 5-phosphate into [H_N2'-^{2}H]NAD^+. The resonances for H_A1' and H_N1' in authentic NAD^+ both appear as doublets between 6.0 and 6.2 ppm, being split by H_A2' and H_N2', respectively (Figure

(23) (a) Jardetzky, O.; Wade-Jardetzky, N. G. *J. Biol. Chem.* **1966**, *241*, 85–91. (b) Sarma, R. H.; Ross, V.; Kaplan, N. O. *Biochemistry* **1968**, *7*, 3052–3062. (c) Sarma, R. H.; Kaplan, N. O. *J. Biol. Chem.* **1969**, *244*, 771–774. (d) Sarma, R. H.; Kaplan, N. O. *Biochem. Biophys. Res. Commun.* **1969**, *36*, 780–788.

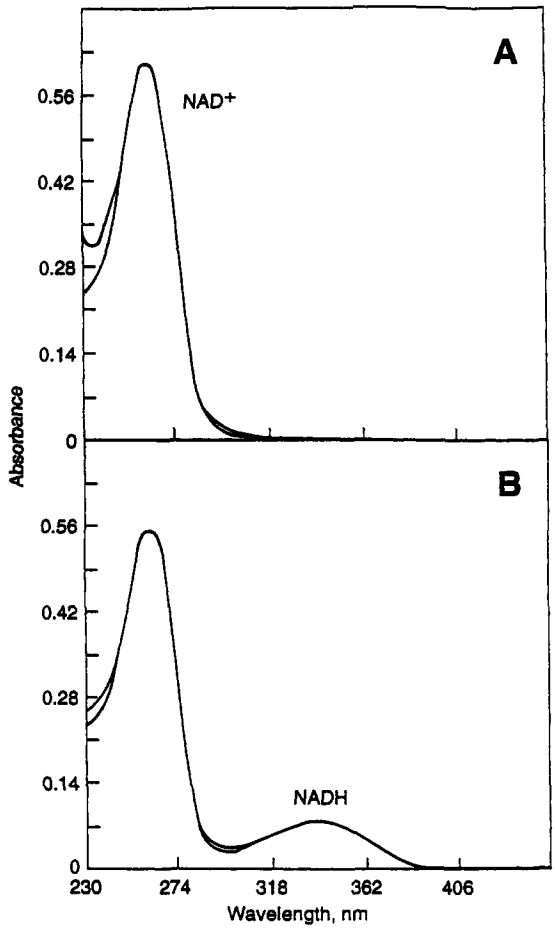

Figure 4. Spectral properties of enzymatically synthesized NAD⁺. In panel A, the absorption spectrum of a commercial preparation of NAD⁺ (Sigma) is compared with that of a mixture of [H_N1'-^{3}H]- and [C_N5'-^{14}C]NAD⁺, synthesized by the method shown in Figure 2. Samples were in 50 mM HEPES, pH 8.0. The same samples are shown in panel B after the addition of ethanol to 10 mM and 0.1 U of alcohol dehydrogenase, followed by incubation for 15 min at 30 °C.

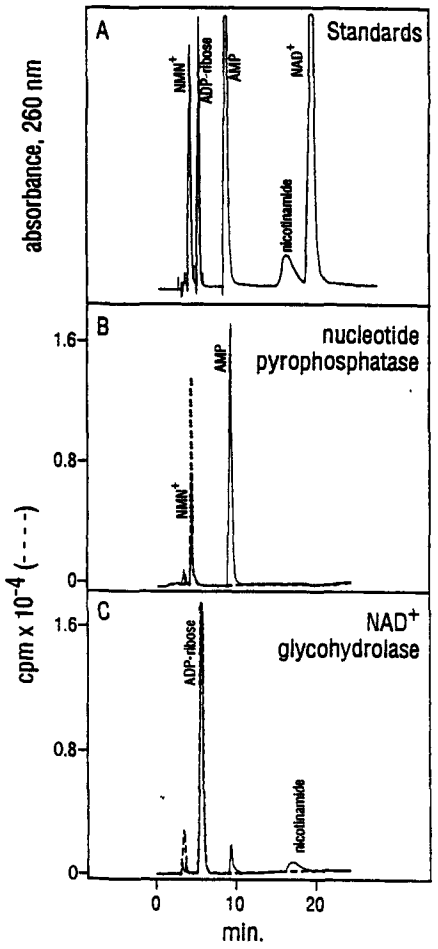

Figure 5. HPLC profile of enzymatic degradation of a mixture of [H_N1'-^{3}H]- and [C_N5'-^{14}C]NAD⁺ with corresponding radioactivity elution profile. For comparison with lower panels, panel A shows elution times of all substrates and products of the NAD⁺ glycohydrolase and nucleotide pyrophosphatase reactions. Panel B shows elution of AMP and NMN⁺, produced by nucleotide pyrophosphatase treatment of NAD⁺, and the corresponding elution of radioactivity with NMN⁺. Panel C shows elution of ADP-ribose and nicotinamide, produced by NAD⁺ glycohydrolase treatment of NAD⁺, and the corresponding elution of radioactivity with ADP-ribose. Both reactions were done at 37 °C in 50 mM potassium phosphate, pH 7.0, with 1 mM unlabeled NAD⁺ as carrier. The amounts of nucleotide pyrophosphatase and NAD⁺ glycohydrolase used were 1 and 0.2 U, respectively.

6). The analogous spectrum of [H_N2'-^{2}H]NAD⁺ shows the H_A1' resonance unchanged, as expected, while approximately 95% of the H_N1' resonance occurs at a single field strength. The H_N1' is not split by H_N2' because deuterium has replaced the proton at the H_N2' position. This demonstrates specific incorporation of deuterium from [2-^{2}H]ribose 5-phosphate into the H_N2' position of NAD⁺ as well as showing that about 95% of the labeled NAD⁺ molecules contain deuterium in the H_N2' position. With the exception of the H_N1' signal, the NMR spectra of authentic and [H_N2'-^{2}H]NAD⁺ are identical in all resonances downfield of the solvent peak. Since the [2-^{2}H]ribose 5-phosphate was made and incorporated into NAD⁺ in analogous fashion to the synthesis and incorporation of [2-^{3}H]ribose 5-phosphate, this result provides evidence for the correct position of label in [H_N2'-^{3}H]NAD⁺.

Discussion

The enzymatic synthesis of NAD⁺ presented here provides good yields of high purity NAD⁺ with specific incorporation of label. The enzymatic synthesis uses glucose as a precursor for the pentose of the NMN⁺ portion of NAD⁺. The biosynthetic method is general since the starting glucose is available, or can be prepared, with radioactive or stable isotope substitutions in a number of positions. This allows introduction of isotopes near the N-glycosidic bond of the NMN⁺ portion of the dinucleotide, the region of interest for kinetic isotope effects with NAD⁺ glycohydrolases and ADP-ribosyltransferases and hydrolases.

Systematic conversion of glucose to 5-phosphoribosyl-1-pyrophosphate (Figure 2) allows direct incorporation of most of the desired labels into this compound. An exception is the synthesis of pentose phosphates labeled at the 2-H-position, for example, [2-^{3}H]pentose phosphate. Conversion of [3-^{3}H]6-phosphogluconic acid to ribulose 5-phosphate involves the formation of a 3-ketohexose which decarboxylates to the 2-ketopentose, ribulose 5-phosphate, and thus causes loss of label introduced as [3-^{3}H]glucose.[21] This problem is circumvented by using the phosphoriboisomerase-catalyzed exchange of solvent tritium into the 2-position of ribose 5-phosphate. This stable precursor provides a direct entry into the reactions of Figure 2. The enzymatic exchange yields an equilibrium mixture of [2-^{3}H]-ribose 5-phosphate and [1-^{3}H]ribulose 5-phosphate. The equilibrium favors ribose 5-phosphate, and only the [2-^{3}H]ribose 5-phosphate is active in the conversion to 5-phosphoribosyl-1-pyrophosphate.

The specificity of the eight enzymes required for the conversion of glucose to NAD⁺ ensures that only the desired starting materials are incorporated into the NAD⁺ product. This is advantageous since many of the commercially available radiolabeled sugars contain significant label which is not incorporated into the product NAD⁺ (Table 2).

It is also possible to synthesize NAD⁺ with the multiple

Table 2. Synthesis of Labeled NAD^+

label and position	starting label[a]	yield from starting sugar or ATP,[b] %	yield from starting label,[c] %
$[H_N1'\text{-}^3H]NAD^+$	[2-^{3}H]glucose	75	53
$[H_N2'\text{-}^3H]NAD^+$	[2-^{3}H]ribose 5-PO_4	85	9
$[H_N4'\text{-}^3H]NAD^+$	[5-^{3}H]glucose	69	13
$[H_N5'\text{-}^3H]NAD^+$	[6-^{3}H]glucose	96	64
$[C_N1'\text{-}^{14}C]NAD^+$	[2-^{14}C]glucose	65	57
$[C_N5'\text{-}^{14}C]NAD^+$	[6-^{14}C]glucose	72	67
$[N_N1\text{-}^{15}N, C_N1'\text{-}^{14}C]NAD^+$	[1-^{15}N]nicotinic acid, [2-^{14}C]glucose	54	53
$[N_N1\text{-}^{15}N, C_N5'\text{-}^{14}C]NAD^+$	[1-^{15}N]nicotinic acid, [6-^{14}C]glucose	88	76
$[C_A8\text{-}^{14}C]NAD^+$	[8-^{14}C]ATP	81	70

[a] Labeled sugars were purchased or produced as described in the Materials section and used without further purification, unless otherwise noted. [b] Yield of NAD^+ from the starting sugar is the yield of purified NAD^+, determined spectrophotometrically, relative to the total glucose or ribose 5-PO_4 present in the initial reaction mixture. In the synthesis of $[8\text{-}^{14}C]NAD^+$ the yield is from added [8-^{14}C]ATP. [c] Yield from starting label is the total radioactivity present in purified NAD^+ relative to the total radioactivity added to the original reaction mixture. In most cases, a substantial fraction of the ^{3}H-labeled sugar was not incorporated into NAD^+ despite efficient conversion of carrier glucose to product. Tritium labeled compounds are prone to radiolysis. However, the degradation products are excluded by the highly specific enzymatic steps used for the synthesis. No attempt was made to characterize the nature of the nonreactive materials. Conversion of commercial [8-^{14}C]ATP to NAD^+ was 70%, reflecting the purity of the [8-^{14}C]ATP preparation.

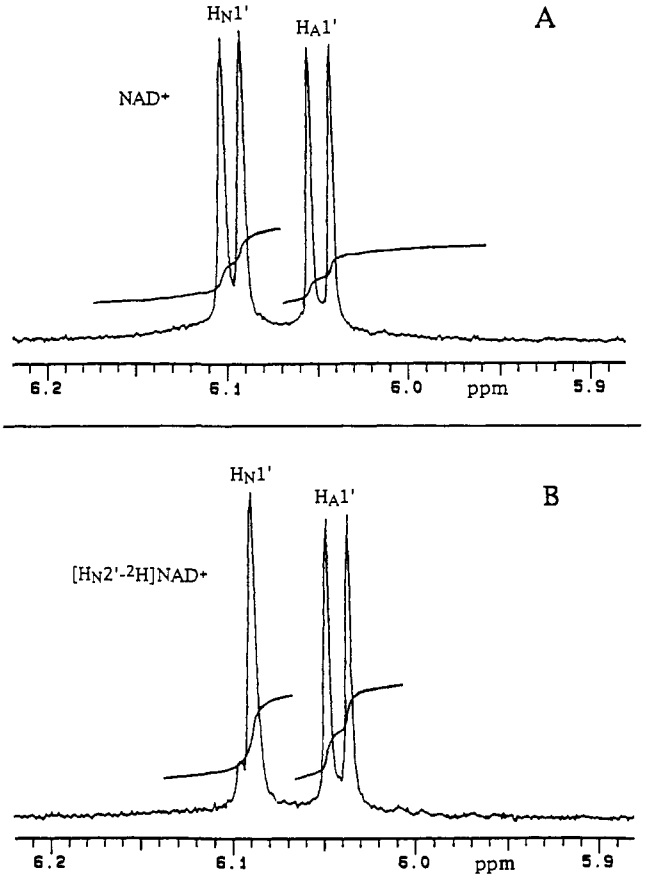

Figure 6. 500-MHz ^{1}H NMR spectra of H_N1' and H_A1' signals of authentic NAD^+ (panel A) and $[H_N2'\text{-}^2H]NAD^+$ (panel B). The H_N1' signal is not split by H_N2' in the $[H_N2'\text{-}^2H]NAD^+$. Peak assignments are according to published spectra.[23] The NAD^+ (5–10 mM) is in ^2H_2O solvent containing 50 mM potassium phosphate, pD 7.4. Chemical shifts are measured relative to the methyl resonance of 3-(trimethylsilyl)-tetradeuterio sodium propionate.

incorporation of heavy atom labels. Synthesis of $[N_N1\text{-}^{15}N, C_N1'\text{-}^{14}C]$- and $[N_N1\text{-}^{15}N, C_N5'\text{-}^{14}C]NAD^+$ is an extension of the reactions in Figure 2 simply by the addition of two labels, [1-^{15}N]-nicotinate and [2-^{14}C]- or [6-^{14}C]glucose, respectively, to the same synthetic mixture. Incorporation of any other label into nicotinic acid would obviously provide the desired label in NAD^+.

Specific incorporation of label into the adenylate of NAD^+ is also possible using the synthetic scheme shown in Figure 2. ATP phosphoryl transfer energy is used to activate the sugars and provides the adenylate of $NaAD^+$. Addition of labeled ATP in step 1 of the scheme in Figure 2 will result in specific incorporation into the adenylate of $NaAD^+$, followed by its conversion to NAD^+ in the usual method by step 2 of Figure 2. However, at the concentrations of reagents used in this enzymatic synthesis, only approximately one-half of the initial ATP would be incorporated. Omission of NAD^+ pyrophosphorylase from the reactions in step 1 of Figure 2 yields $NaMN^+$ with desired labels which can then be converted with good efficiency to $NaAD^+$ by NAD^+ pyrophosphorylase in the presence of labeled ATP. For example, synthesis of $[C_A8\text{-}^{14}C]NAD^+$ required conversion of $NaMN^+$ to $NaAD^+$ in the presence of [8-^{14}C]ATP. This protocol provides $NaAD^+$, with label in the adenylate portion of the molecule, which can then be converted to NAD^+ with NAD^+ synthetase. With equimolar $NaMN^+$ and ATP (1 mM each), overall chemical conversion and recovery of purified $[C_A8\text{-}^{14}C]NAD^+$ was 81% (Table 2).

Synthesis of adenylates with label in both the ribosyl and adenine rings has been documented.[13,14,24] Combination of these methods for labeled ATP synthesis, together with the use of labeled glucose and/or nicotinic acid, permits synthesis of NAD^+ with labels in nearly all nonexchangeable positions of the molecule.

Enzymatic synthetic methods are finding increased applications in bioorganic chemistry. The synthesis of specifically labeled NAD^+ molecules from glucose provides an example of the utility of these methods. Labeled NAD^+ molecules are of increasing interest for investigations into the still-expanding variety of ADP-ribosylation reactions. In addition, the compounds are likely to be useful in investigation of remote isotope effects on the family of enzymes which use NAD^+ as an electron transfer agent.

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