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Analytical Biochemistry 325 (2004) 62–67
ANALYTICAL BIOCHEMISTRY
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Synthesis and utility of ^{14}C-labeled nicotinamide cofactors☆

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Received 30 May 2003

Abstract

A new method for the synthesis of the reduced form of β-nicotinamide [U-^{14}C]adenine dinucleotide 2′-phosphate ([Ad-^{14}C]NADPH) is presented. The present synthesis results in a radioactive material with a specific activity that is greater than 220 mCi/mmol. This method could easily be adapted for syntheses of ^{14}C-labeled NADH, NADP$^+$, or any nicotinamide cofactors with radiolabels in other positions. Since these cofactors are so ubiquitous, the use and applications of such labeled material has broad implications. The utility of the labeled cofactor for determination of substrates for nicotinamide-dependent enzymes in the nano- to femtomole scale, in alternative enzymatic assays, and in kinetic isotope effect studies is discussed.

Keywords: Carbon fourteen; Radioactive labeling; Labeled nicotinamide; Reverse-phase; HPLC; Nicotinamide synthesis; NADPH; Glucose dehydrogenase; Creatine phosphate kinase; NAD kinase

Several approaches toward synthesizing labeled NADPH have been reported to date. As experimental approaches using radiolabels vary, so must the isotope or its position. Commonly, nicotinamide cofactors have been labeled with ^{2}H, ^{3}H, ^{14}C, or even ^{32}P [1–7]. These labels are either proximal (the nicotinamide ring) or remote to the site where redox chemistry occurs (in the adenosine or ribose moieties or, for ^{32}P, in the phosphate ester linkages). Different methodologies for generating radiolabeled cofactors range from the use of mice fed with radiolabeled precursor as vectors [1] to the use of Baker's yeast grown in labeled media [1,4] to biogenesis using adenosine triphosphate (ATP)[1] and nicotinamide mononucleotide (NMN) with a crudely purified enzyme fraction [8]. Many of these methods result in low yields and are relatively expensive. Here, we report a high-yield synthesis that results in adenosine-labeled [Ad-^{14}C]NADPH, whose specific radioactivity is equivalent to that of the starting material, [Ad-^{14}C]NAD$^+$.

Hereto, syntheses of remotely labeled nucleotides have been adapted (e.g. [9,10]) from the 1954 procedure of Wang et al. [2,11], which described a method for the synthesis of ^{14}C-labeled NADPH. Their reported synthetic yield was 42% after a 32-h synthesis, where ATP was added in intervals. The current method improves upon this synthesis by an ATP recycling system, which minimize the inhibitory effect of ADP and pushes the reaction toward completion. The synthetic strategy presented here has a much higher yield (>85%) in a 2-h reaction time.

Such remotely labeled cofactor enables several important applications. For example, it is of great interest to establish a methodology that allows simple and accurate determination of substrates in the pico- to femtomole scale [12,13]. Traditional methods, such as UV spectroscopy, are not sensitive enough to measure such minute concentrations and therefore more sensitive techniques need to be employed. Malaisse and co-workers [12] have established a methodology to

☆ This work was supported by NIH R01 GM65368-01 and NSF CHE-0133117.

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[1] *Abbreviations used:* NAD$^+$, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form; NADP$^+$, β-nicotinamide adenine dinucleotide 2′-phosphate; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate, reduced form; [Ad-^{14}C]NAD$^+$, β-nicotinamide [U-^{14}C]adenine dinucleotide; [Ad-^{14}C]NADP$^+$, β-nicotinamide [U-^{14}C]adenine dinucleotide 2′-phosphate; ATP, adenosine triphosphate; NMN, nicotinamide mononucleotide; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; GDH, glucose dehydrogenase; CP, creatine phosphate; CPK, creatine phosphate kinase; GmDH, glutamate dehydrogenase; LSC, liquid scintillation counter; DHFR, dihydrofolate reductase; KIE, kinetic isotope effect.

0003-2697/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2003.10.027

determine very low NAD(P)H concentrations using radiolabeled α-ketoglutarate and glutamate dehydrogenase (GmDH). They also assayed several substrates of NADPH-dependent enzymes using this methodology by coupling their NAD(P)H production to the reaction of GmDH to generate radiolabeled glutamate, which acts as a secondary or indirect reporter of the original substrate concentration. However, such methodology has the disadvantage of relying on an intermediate to report substrate concentrations. Since nicotinamide-containing cofactors are much more ubiquitous than glutamate, it can be used directly to determine many compounds that serve as substrates for nicotinamide-dependent enzymes. We propose that by using radiolabeled NADPH similarly sensitive results can be obtained without the use of an intermediate. Additionally, [Ad-^{14}C]NADPH has potential utility in enzymatic assays and in kinetic isotope effect studies as discussed below.

Materials and methods

Materials

β-Nicotinamide [U-^{14}C]adenine dinucleotide (50 μCi) was purchased from Amersham Pharmacia as the ammonium salt solution at pH 5 in 2% EtOH (specific radioactivity >220 mCi/mmol). ATP, glucose-6-phosphate (G-6-P), NAD^+ kinase (from chicken liver), glucose-6-phosphate dehydrogenase (G-6-PDH) (from Baker's yeast), creatine phosphate (CP), creatine phosphate kinase (CPK), glucose, glucose dehydrogenase (GDH), and NADPH were from Sigma. Tris base, $MgCl_2$ (hexahydrate), and Microcon YM-30 centrifugal filters used for the removal of enzymes were from Fisher. Analysis was performed via HPLC (Agilient 1100 Series equipped with a diode array detector) with a reverse phase C-18 column (Discovery C-18; 250 × 4.6 mm; Suppleco). Flo-LSC (Packard Liquid Flow Analyzer 500TR Series) was used for detection of radioactive material. Ultima-Flo AP flow scintillation cocktail was from Packard (now Perkin–Elmer Biosciences). Semi-preparative purification was performed via HPLC (Agilient 1100 Series equipped with a multiwavelength detector) with a reverse phase C-18 column (Discovery C-18, 250 × 10 mm; Suppleco).

[Ad-^{14}C]NADPH synthesis

[Ad-^{14}C]NAD^+ solution (500 μL; 12.5 μCi; 570 μmol) was pipetted into a 1.5-mL microcentrifuge tube that was used as the reaction vessel. Then, 15 mg Tris base (250 mM), 44 mg ATP (160 mM), 9 mg G-6-P (60 mM), and 20 μL 0.5 M $MgCl_2$ (50 mM) were added to a total volume of 520 μL. The pH was adjusted to 6.5 with 1 M HCl. Final concentrations are in parentheses.

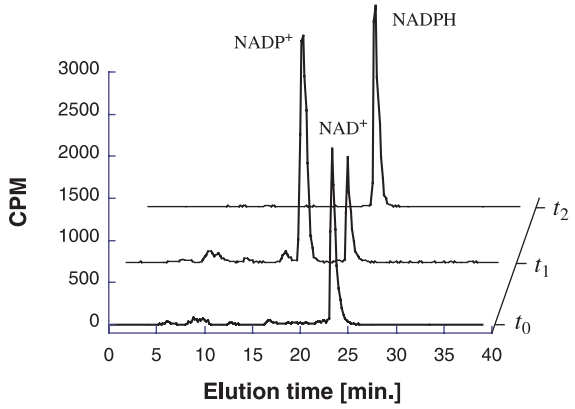


Fig. 1. HPLC-flow detector radiograms following the enzymatic synthesis of [Ad-^{14}C]NADPH. t_0, Reaction mixture before adding enzymes; t_1, reaction mixture at 75% conversion after adding the NAD^+ kinase; t_2, reaction mixture after completion of the reduction.

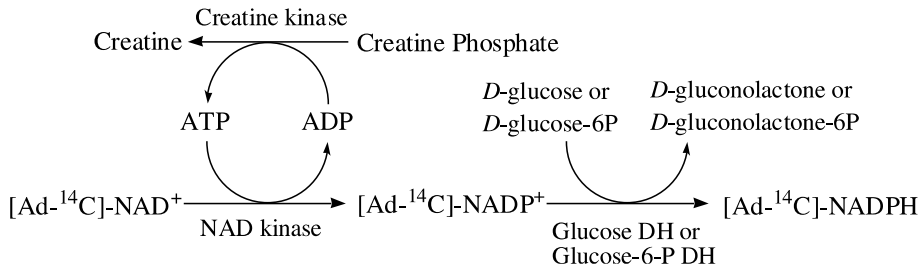
The reaction was initiated by adding NAD^+ kinase (50 units) and was allowed to proceed at 35 °C for 3–4 h until ~75% conversion was reached as determined by HPLC radioactivity analysis (Fig. 1, t_1). The pH was then adjusted to 8.0–8.3 with ~10 μL 10 M NaOH, and G-6-PDH (~50 units) was added and allowed to react for approximately 45 min at room temperature. An aliquot was analyzed via HPLC and Flo-LSC to ensure completion (see Fig. 1, t_2 and Results and discussion below). The enzymes were filtered out with a Microcon YM-30 centrifugal filter device at 4 °C. The synthesized [Ad-^{14}C]NADPH was purified via HPLC, lyophilized, and stored at −80 °C [14].

Creatine recycling system

An alternate method for this synthesis consisted of recycling adenosine diphosphate (ADP; product of the NAD^+ kinase reaction) to ATP. ATP regeneration was achieved by the addition of CP and CPK. The reaction was prepared as described above with the exception of adding significantly less ATP (2 mM) and adding CP in excess of the radioactive NAD^+ (15.6 mM). CPK (~340 units) was added before the initiation of the reaction by NAD^+ kinase. This reaction was incubated for ~30 min at 35 °C for complete phosphorylation of the NAD^+. Reduction of the nicotinamide ring followed the phosphorylation and proceeded as described above, using glucose and GDH (~50 units), instead of the G-6-P and G-6-PDH. Scheme 1 outlines this recycling system.

Analytical methodology

The course of the phosphorylation and reduction reactions were followed via a reverse phase HPLC equipped with an analytical column and a UV diode array detector. In conjunction with UV absorbance detection, a Flo-LSC was used for detection of



Scheme 1. Synthetic strategy.

radioactivity. The analytical HPLC method consisted of a 0.1 M KH_2PO_4, pH 6.0, mobile phase with a MeOH gradient [14]. Using this method, $NADP^+$ elutes at $t_R = 18.5$ min, NAD^+ at $t_R = 25.7$ min, NADPH at $t_R = 26.6$ min, and NADH at $t_R = 33.0$ min. Sample analytical radiochromatograms of reaction progress are presented in Fig. 1.

Semipreparative purification and preservation

The filtered reaction mixture from the [Ad-^{14}C]NADPH synthesis was purified via HPLC using a reverse phase semipreparative column (Discovery C-18; 250×10 mm). The mobile phase for this separation was 200 mM NaCl with 1 mM Tris/HCl adjusted to pH 8.2. A MeOH gradient was introduced to improve resolution and peak appearance; fractions containing [Ad-^{14}C]NADPH (a sharp radioactive peak at 17 min) were pooled and lyophilized. Using this purification and preservation method, synthesized NADPH has been shown to be 97% pure for at least 26 months [14].

Results and discussion

[Ad-^{14}C]NADPH synthesis

[Ad-^{14}C]NADPH was synthesized from [Ad-^{14}C]NAD^+ using NAD^+ kinase and G-6-PDH along with their proper substrates. The overall yield of the [Ad-^{14}C]NADPH reaction prior to lyophilization was 85%. In repeated syntheses, it was noted that there was loss of product upon lyophilization. We believe that these losses (between 2 and 10%) were due to the resulting flocculent lyophilized powder. This suggestion is in accordance with the findings of Jeong and Gready [15] for the synthesis of deuterium-labeled [4-^{2}H] NADPH, and indeed, a higher salt in the HPLC's mobile phase (200 mM NaCl) helped to settle the lyophilizate, keeping product loss to <2%.

The starting material NAD^+ and the final product NADPH do not resolve well using HPLC methods (cf. Fig. 1). Consequently, remaining [Ad-^{14}C]NAD^+ should be removed prior to purification. To achieve this goal, the following methods were tested. (1) An $NADP^+$-specific dehydrogenase was used to reduce the phosphorylated cofactor so that the equilibrium of the NAD^+ kinase was shifted toward products, resulting in complete consumption of [Ad-^{14}C]NAD^+. (2) Creatine phosphate and its specific kinase were used to recycle the ATP. This enabled "pushing" the phosphorylation to completion with a large excess of creatine phosphate and speeding up the reaction by eliminating the inhibitory effect of ADP. (3) Remaining [Ad-^{14}C]NAD^+ was reduced with yeast alcohol dehydrogenase to generate [Ad-^{14}C]NADH, which is easily resolved by HPLC. This last method wasted the expensive starting material, so methods 1 and 2 were preferred. The second method has an advantage over the first method due to a shorter reaction time (1 h versus 4 h).

Oxidized nicotinamides are known to be more stable at pH 4–7 [16,17] while the reduced cofactor is more stable at a higher pH (8–10) [17–19]. In the one-pot synthesis described above, these stability differences had to be taken into consideration. Throughout the phosphorylation, the pH of the reaction was 6.5. The pH was adjusted to ~8 prior to the addition of the reducing enzyme, but was kept below 8.5 since the rates of the phosphorylation and reduction are both slower at a high pH. The enzyme-catalyzed reduction rate was controlled by changing the enzyme concentration. When using about 50 units of G-6-PDH the reaction is usually completed in 45 min (by HPLC analysis).

Creatine recycling system

Recycling the ATP with CP and CPK improved the overall synthesis in that significantly less ATP was used and a shorter reaction time was needed. Since ADP is an inhibitor of NAD^+ kinase, having a significant excess of this nucleotide in the reaction mixture most likely inhibits the phosphorylation step. Lowering the ATP concentration and adding CP in excess (CP is not an inhibitor of NAD^+ kinase) allowed for a constant steady state concentration of ATP. An even more significant effect is that the excess of CP draws the equilibrium toward products. This method allowed for near completion of the phosphorylation (~98%) so that remaining amounts of NAD^+ were negligible. Therefore, using this recycling system, GDH ($NADP^+$/NAD^+ depen-

dent) could be used without the concern of wasting the radioactive starting material. Any amount of NADH that was produced using this method was easily separated during purification.

When employing either of the synthetic methods described above, [Ad-^{14}C]NADPH with a specific radioactivity greater than 220 mCi/mmol is generated. This specific activity is equal to that of the starting material. This synthesis can easily be adapted to synthesize NADH or NADP$^+$ simply by eliminating the phosphorylation or the reduction steps, respectively. Additional flexibility is afforded by this method in the context of alternative labeling patterns. The starting material, [Ad-^{14}C]NAD$^+$, can be substituted with a nucleotide of any desired radioactive or stable isotope and/or with an isotope labeling in an alternative position.

Product purity

To test the purity of the final, lyophilized product, it was resolubilized and analyzed by HPLC followed by fraction collection (1-min fractions). Each fraction was analyzed by a liquid scintillation counter for 5 min and the resulting radiogram is presented in Fig. 2, t_0. This method is significantly more sensitive than radioactivity flow detection due to its large total counts and the external standard DPM correction. This analysis showed that more than 98% of the radioactivity was in the [Ad-^{14}C]NADPH while less than 1% was in its oxidation product ([Ad-^{14}C]NADP$^+$). Furthermore, the resolubilized [Ad-^{14}C]NADPH was consumed by the enzyme dihydrofolate reductase (DHFR) in the presence of large excess of dihydrofolate. Fig. 2, t_∞ presents the HPLC–LSC analysis of that reaction, which resulted in more than 98% of the radioactivity in the [Ad-^{14}C]NADP$^+$, about 0.4% at 3 and 9 min (consistent with ADP and AMP, respectively), and less than 1% at the NADPH peak (25 min). This last peak might be the result of noncomplete DHFR oxidation due to final NADPH concentration below the K_m of the enzyme (1 μM [20]).

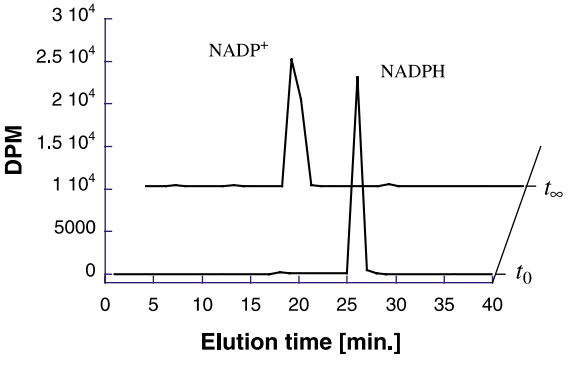


Fig. 2. HPLC–LSC radiograms following the DHFR-catalyzed oxidation of [Ad-^{14}C]NADPH. t_0, Reaction mixture before adding DHFR; t_∞, reaction mixture after completion of the oxidation.

Applications

Three typical applications of [Ad-^{14}C]NADPH and other remotely labeled nicotinamide cofactors are discussed below.

Alternative enzymatic assay. Initial velocity experiments of nicotinamide-dependent enzymes are often accomplished by following the absorbance changes at 340 nm. The sensitivity of these spectrophotometric experiments is limited to cofactor concentrations in the micromolar range (0.006 absorption units using $\varepsilon = 6200\,M^{-1}\,cm^{-1}$). A better sensitivity (in the nanomolar range) has been reached by Aiso et al. [21] using HPLC–electrochemical detection for a dihydrofolate reductase assay. An even higher sensitivity can be achieved while using ^{14}C-labeled cofactor to follow the reaction progress with HPLC–radioactivity detection analysis. For this method, the reaction mixture is injected into the HPLC system at various time points and the reaction rate can be calculated from the radioactivity trace. The utilization of radiolabeled nicotinamide cofactors for initial velocity experiments has the advantage of acquiring measurements at very low concentrations (nanomole to picomole), which are often below the K_m of the enzymatic system. This affords accurate examination of the enzyme's second-order constant V/K. The importance of utilizing [Ad-^{14}C]NADPH as the radioactive tracer is that no kinetic isotope effect is anticipated to affect the measurements, as the effect of remote ^{14}C on binding and kinetics is neglectable [22]. Due to the ubiquitous nature of nicotinamide cofactors, such an assay has many applications with a wide variety of enzymes.

Competitive kinetic isotope effects (KIEs). Tritium KIE is an important tool in studies of enzyme mechanisms. The tritium KIE can be used together with the more common deuterium KIE to elucidate the intrinsic KIE [23] or to investigate the contribution of hydrogen tunneling and coupled motion to the enzymatic reaction [24–26]. Since tritiated materials are commonly labeled with a trace amount of tritium, a competitive experiment is commonly used. In such an experiment, the tritiated and nontritiated substrates, or cofactors, are mixed and the KIE is calculated from the ratio of the tritiated and nontritiated products [22,24]. The only way to reach the high sensitivity required in this type of experiment is to follow the nontritiated compound by remote labeling such as [Ad-^{14}C]NADPH. Hereto, such experiments were carried out by labeling the specific substrate for the enzyme of interest. The synthesis of radiolabeled cofactor enables such an experiment with any nicotinamide-dependent enzyme and eliminates the need for specific labeling of substrates.

Detection of substrates at nano- to femtomole quantities. The [Ad-^{14}C]NADPH can be used to detect very small quantities of substrates of NADPH-dependent

enzymes. The [Ad-^{14}C]NADPH should be used in excess over the unknown amount of substrate (if the entire radioactivity is consumed, it was not in excess). The appropriate enzyme should be added in large enough quantity to assure fast completion of the reaction (despite low concentrations of both substrate and cofactor). The amount of [Ad-^{14}C]NADP$^+$ produced can then be determined by HPLC followed by radioactivity analysis and the known specific radioactivity of the cofactor. For example, in an assay reported by Sener and Malaisse [12], a standard calibration curve was constructed for [U-^{14}C]α-ketoglutarate. The concentrations of the unknown NAD(P)H were very small and the labeled substrate, [U-^{14}C]α-ketoglutarate, was in excess. GmDH was added and an incubation period of 30 min was allowed for reaction completion. The reaction was halted and small aliquots of the reaction mixture were analyzed to measure radioactivity. Ultimately, the number of moles per sample of L-[U-^{14}C]glutamate product was determined. Such an assay allowed detection of picomole amounts of various substrates by producing NADPH through the oxidation of the unknown substrate with excess of NADP$^+$ coupled to the reduction of the NADPH by [U-^{14}C]α-ketoglutarate and GmDH [12]. The limit of the detection is therefore established by the specific radioactivity of the original radioactive reagent. The specific radioactivity of the [Ad-^{14}C]NADPH synthesized by the procedure described here is similar to that of the α-ketoglutarate used to measure picomole amounts of NADPH [12]. Consequently, the sensitivity of an assay using the 220 mCi/mol [Ad-^{14}C]NADPH should be at the nano- to picomole range for various substrates of NADPH-dependent enzymes. Femtomolar amounts of substrates have been detected [13] using starting material with a much higher specific activity (54.7 Ci/mmol of tritium). Similarly, NAD(P)$^+$ or NAD (P)H can be used to measure similar amounts, simply by using the commercially available tritiated NAD$^+$ (nicotinamide [2,5,8-^{3}H]adenine dinucleotide; Amersham Biosciences) with a specific activity between 30 and 70 Ci/mmol as starting material. ^{32}P-NAD$^+$ is also commercially available (Amersham) with a specific radioactivity of ~1000 Ci/mmol, further emphasizing the utility and versatility of such assays conducted with labeled, ubiquitous cofactors rather than individual substrates. Importantly, as in the case of [Ad-^{14}C]NADPH, all these cofactors are radiolabeled at a position that should not lead to significant equilibrium or kinetic isotope effects. Establishing methodology for these sensitive assays that utilize the more ubiquitous nicotinamide cofactors instead of the secondary indicator (e.g., [U-^{14}C]α-ketoglutarate in [12]) has numerous potential applications in the determination of small substrate quantities (nanomole to femtomole) for nicotinamide-dependent enzymes.

Finally, remote labeling of nicotinamide cofactors is not limited to radioactive isotopes. Adenine-labeled ^{13}C- and ^{15}N-NAD$^+$ can be synthesized from the commercially available adenosine and adenosine monophosphate (Cambridge Isotope Laboratories, Inc.). Using the above procedure, ^{13}C- and ^{15}N-NAD$^+$ can be converted into NADPH or related derivatives and used in various NMR and ENDOR experiments.

Acknowledgments

The authors thank Lin Wang for his assistance.

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