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A HIGH YIELD MICROSCALE ENZYMATIC SYNTHESIS AND PURIFICATION OF ^{14}C-LABELED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADP$^+$)[1]

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Summary

Uniformly labeled (U) ^{14}C nicotinamide adenine dinucleotide phosphate (NADP$^+$) was synthesized by phosphorylating [U-^{14}C]nicotinamide adenine dinucleotide (NAD$^+$) in the presence of immobilized NAD$^+$ kinase. The 15 μCi (600 μL) synthesis consistently achieved yields between 80% and 85% and radiochemical purities greater than 95%. The [U-^{14}C]NADP$^+$ was purified by high performance anion-exchange chromatography using a gradient elution of ammonium bicarbonate. This procedure may be applicable to the synthesis of other charged, UV-absorbing products of enzyme-catalyzed reactions.

Keywords: ^{14}C-Nicotinamide Adenine Dinucleotide Phosphate, Microscale Enzymatic Synthesis

Introduction

One of the most sensitive ways of measuring the dissociation constant and stoichiometry of an enzyme-substrate complex is through the use of radioactive binding experiments.[2,3] NADP$^+$ is a substrate for many dehydrogenase reactions, including glucose-6-phosphate dehydrogenase (G6PD) isolated from human erythrocytes.[4] Because [U-^{14}C]NADP$^+$ is not commercially available, our experiments to measure the binding of NADP$^+$ to G6PD required that it be synthesized.

[U-^{14}C]NADP$^+$ has been previously prepared by phosphorylating [U-^{14}C]NAD$^+$ with ATP in the presence of soluble NAD$^+$ kinase. The [U-^{14}C]NADP$^+$ was then utilized in an unpurified form.[5,6] Unpurified [U-^{14}C]NADP$^+$ appears unsuitable for our purposes because G6PD and NADP$^+$ concentrations are determined spectrophotometrically, and hence, contamination of these solutions by unreacted ATP or side products may give erroneous results in our binding studies. Furthermore, our unpurified [U-^{14}C]NADP$^+$ preparation contained ATP and ADP, which are inhibitors of G6PD with respect to NADP.[7] A tritiated version of NADP$^+$ that is selectively labeled at the 4-position may undergo an exchange reaction on binding to

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G6PD in the presence of some alternate substrates.[8] This might result in the loss of the tritium label on $NADP^+$. In light of prior difficulties in measuring $NADP^+$ binding to G6PD, it was decided that a purified version of [U-^{14}C]$NADP^+$ was needed to insure accurate, unambiguous and reproducible binding studies.[7]

Materials

Amersham supplied the [U-^{14}C]NAD^+ (50 μCi in 2mL; Product CFA.497). Sigma Chemical furnished the NAD^+ kinase (250 units; Product N-8882; Lot 118F7185), sodium azide, potassium phosphate monobasic, ammonium bicarbonate, EDTA, sodium acetate, β-mercapto-ethanol, 3-[*N*-morpholino]propanesulfonic acid (MOPS) and ATP (Product A-2382; Lot 88F-7075). J. T. Baker Chemical supplied the magnesium chloride and Maxiflour LCS counting fluid, while Eastman Kodak furnished the glycine ethyl ester and Bio-Rad Laboratories supplied the Affi-Gel 15.

Methods

Preparation of the Immobilized NAD^+ Kinase. Approximately 400 mg of Affi-Gel 15 was thoroughly washed by filtration with glass-distilled water and a 50-80 mg aliquot placed in each of five custom made glass vials with the dimensions of a 1.5 mL Eppendorf tube. Two hundred and fifty units of NAD^+ kinase was dissolved in 1.0 mL of 0.15 M MOPS buffer (pH 6.8) and 200 μL aliquots were added to each glass vial. A 7 mm x 2 mm Teflon-coated stirring bar was also placed in each vial and the gel beads were suspended throughout the mixture by gentle stirring over three hours. Thirty microliters of 1.0 M glycine ethyl ester (pH 8.0) was then added to block any unreactive sites on the gel beads.[9] The reaction proceeded for 30 minutes. The supernatant was removed from the settled gel beads with a 1.0 mL HPLC syringe until the gel beads appeared "dry." Fifty microliters of 0.15 M MOPS was added to each vial and the beads were resuspended. The supernatant was removed as before. This washing procedure was repeated 4 times to ensure the removal of any free glycine ethyl ester and unreacted NAD^+ kinase.

Reaction of [U -^{14}C]NAD^+ with ATP. Six hundred microliters (15μCi) of [U-^{14}C]NAD^+ was concentrated to 200 μL by passing a gentle stream of dry nitrogen gas over the solution for an hour.[10] The 200 μL sample of concentrated [U-^{14}C] NAD^+ was then added to the vial containing the dry gel beads. To this mixture was added 3.0 μL of 0.5 M $MgCl_2$ and 30 μL of 40 mM ATP in 0.15 M MOPS (pH 6.8). The gel beads were suspended by gently stirring the solution for 2 h and 45 min. The mixture was then allowed to settle and the supernatant removed as before and placed in an identically shaped vial. The gel beads were washed 5 times with 50 μL aliquots of 0.15 M NH_4HCO_3 and the washings pooled with the supernatant. Analysis showed that greater than 99% of the radioactivity was recovered from the gel beads.

HPLC Separation of the Reaction Mixture. Two 250 x 4.6mm ID SynChronpak AX-100 anion exchange columns (SynChrom, Lafayette IN) were linked in tandem and connected to a Hewlett-Packard 1090 HPLC system. The columns were thoroughly flushed with 0.45 micron filtered

glass-distilled deionized water. The combined supernatant and washings (~450 µL) were loaded on a 1.0 mL injection loop between 400 µL and 150 µL of glass-distilled water. The separation was monitored at 254 nm. A typical profile appears in Figure 1.

The elution was effected at time zero with a 15 min linear gradient of glass-distilled water versus 2M NH_4HCO_3. Two molar NH_4HCO_3 was then passed through the columns for 5 min to remove any remaining UV-absorbing compounds. The columns were then cleared of ammonium bicarbonate with a linear gradient of 2M NH_4HCO_3 versus a solution of 50mM KH_2PO_4 and 0.04% NaN_3 (pH 6.8) for 16 min and followed by a 50mM KH_2PO_4 and 0.04% NaN_3 (pH 6.8) solution for 44 min. A linear gradient of 50mM KH_2PO_4 and 0.04% NaN_3 (pH 6.8) versus glass-distilled water was used for 40 min to remove the remaining KH_2PO_4 and NaN_3. The columns were then flushed with glass-distilled water for 3 h to remove any remaining salts from the column.

The [U-^{14}C]NADP$^+$ peak was collected in a 10 mL glass vial, immediately frozen in liquid nitrogen, and allowed to lyopholize overnight. After lypholization, the sample was rehydrated with 2.00 mL of buffer containing 0.05 M EDTA, 0.05 M sodium acetate, and 0.1% β-mercaptoethanol (pH 7.0). Two 5 µL samples were taken with positive displacement pipettes and placed in 7 mL glass liquid scintillation vials. Five milliliters of Maxiflour was added to each vial and the samples were counted on LKB 1217 liquid scintillation counter. Disintegrations per minute were measured on successive days until highly reproducible results were obtained. The fifth day results were usually used in the yield calculations.

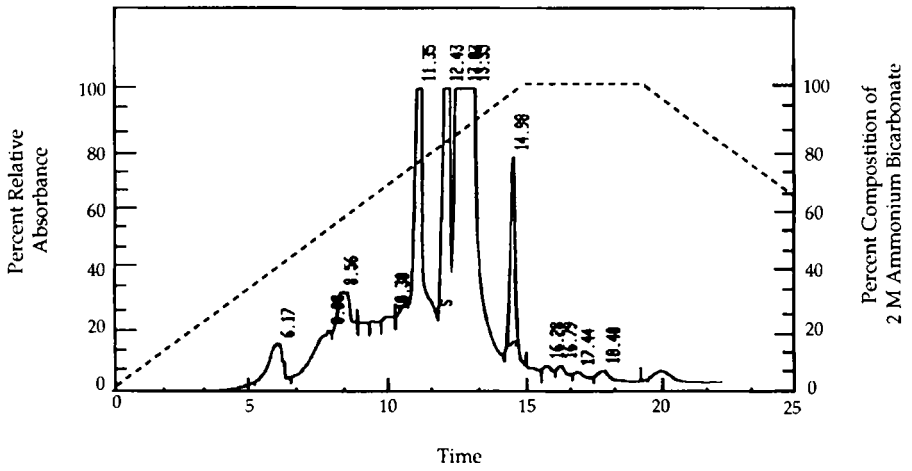

Figure 1: HPLC separation profile: Retention times in minutes (flow rate 1mL per min) [U-^{14}C]NAD$^+$ 8.56, [U-^{14}C]NADP$^+$ 11.35, AMP 12.43, ADP 13.03, ATP 13.35. HP 3392A Integrator settings: zero 15, attenuation 8, chart speed 0.5, threshold 7, peak width 0.04, area reject 100000. Peaks were identified by their co-elution times with standards. The peaks are truncated because of the high amplication used to show the separation of the small [U-^{14}C]NAD$^+$ peak.

Results and Discussion

The high cost of commercially available [U-^{14}C]NAD$^+$ makes it important to achieve high yields. Our process has consistently achieved 80-85% yields with less than 4% of the

[U-^{14}C]NAD^+ remaining unreacted. Radioactive purities greater than 95% were measured by reisolating a purified sample of [U-^{14}C]$NADP^+$ using the same HPLC setup.

AMP contamination of the $NADP^+$ has been a significant problem in some commercial preparations and has also hampered enzymatic assays.[11] AMP contamination may present similar problems with procedures that rely on the synthesis of [U-^{14}C]$NADP^+$ from [U-^{14}C]NAD^+.[12] Analysis of the purified $NADP^+$ peak showed that it was not contaminated by any of the other UV light-absorbing peaks, including NAD^+, AMP, ADP, and ATP. The sample of $NADP^+$ may, however, contain trace amounts of non-UV light-absorbing species including: MOPS (less than 0.0023 M), $MgCl_2$ (less than 0.0008 M), and KH_2PO_4 (less than 0.0025 M). These concentrations are reduced by three to four orders of magnitude when the sample of [U-^{14}C]$NADP^+$ is diluted into buffers used for binding studies. These small concentrations of non-UV absorbing species are not likely to affect our binding experiments.

Other HPLC purification techniques were considered, but anion exchange chromatography provided superior resolution of the reaction products and the capacity to purify at least 15 μCi of [U-^{14}C]$NADP^+$ per run.[13,14] The ammonium bicarbonate used in the separation of [U-^{14}C]$NADP^+$ sublimes under lypholization, which eliminates the need for a desalting step. We did not observe the significant drop in yields during the lypholization of ammonium bicarbonate that others have reported.[15]

Our experience strongly suggests that it should be feasible to scale up the isolation process to a level of 50 to 200 μCi with little difficulty. The present system of linking two columns in series affords adequate resolution at 50 μCi to separate [U-^{14}C]$NADP^+$ from the adjacent AMP peak. Larger amounts of material may be separated by linking more ion-exchange columns and adjusting the salt gradient accordingly.

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