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SYNTHESIS OF 4R- AND 4S-TRITIUM LABELED NADPH FOR THE DETERMINATION OF THE COENZYME STEREOSPECIFICITY OF NADPH:PROTOCHLOROPHYLLIDE OXIDOREDUCTASE

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Summary. A rapid and easy method for the production of both the 4R and 4S tritium labeled isomers of either NADH or NADPH has been developed. The method requires the use of only a single labeled compound (D-[1-^{3}H]glucose), and two enzymes (glucose dehydrogenase from *Bacillus* sp. and alcohol dehydrogenase from *Thermoanaerobium brockii*) which are specific for the pro S and pro R hydrogens, respectively, of either NADH or NADPH. The 4R and 4S tritium labeled isomers of NADPH have been used to determine that NADPH:protochlorophyllide oxidoreductase from etiolated wheat was specific for the pro S hydrogen of NADPH.

Most oxidoreductases requiring nicotinamide coenzymes stereospecifically remove either the pro R or the pro S hydrogen from C4 of the nicotinamide ring of either (but usually not both) NADH or NADPH (1). Studies to determine the coenzyme stereospecificity of an oxidoreductase therefore require the use of stereospecifically reduced, tritium labeled NADH and/or NADPH. Since [4-^{3}H]NAD$^+$ is commercially available, both stereoisomers of [4-^{3}H]NADH can be generated using appropriate enzymes with unlabeled substrates. However, [4-^{3}H]NADP$^+$ is not available commercially, and neither are most appropriately labeled enzyme substrates which could be used to produce stereospecifically labeled NADPH (for example the substrate of ADH , [1-^{3}H]ethanol). One reported method (2) for the preparation of [4R-^{3}H]NADPH required the synthesis of [2-^{3}H]isocitrate from triethyloxalosuccinate and [^{3}H]$NaBH_4$. We have employed one of the few appropriately labeled substrates available (the substrate of GDH, D-[1-^{3}H]glucose) to synthesize both stereoisomers of [4-^{3}H]NADPH, and used them to study the nicotinamide coenzyme stereospecificity of the NADPH:protochlorophyllide oxidoreductase (EC 1.6.99.1) from etiolated wheat. This enzyme has been described by Griffiths (3,4) who also first determined its requirement for NADPH (5).

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Abbreviations used: ADH, alcohol dehydrogenase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; Chlide, chlorophyllide; GDH, glucose dehydrogenase; Pchlide, protochlorophyllide; PCR, NADPH:protochlorophyllide oxidoreductase.

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METHODS

Preparation of labeled nicotinamide coenzymes. [4-^{3}H]NAD^+ (80 Ci mol^{-1}; Amersham) was purified on Sephadex G-25 (Pharmacia). The labeled NAD^+ was eluted very near the void volume, well separated from lower MW (labeled) degradation products. Attempts to convert it to [4-^{3}H]$NADP^+$ by the enzyme NAD^+ kinase (Sigma) and ATP led to very low yields (results not shown). [4-^{3}H]$NADP^+$ was synthesized, however, by incubation of 10 mL of 0.5 mM $NADP^+$, 0.5 mM D-[1-^{3}H]glucose (20 Ci mol^{-1}; New England Nuclear), and 10 units of GDH from *Bacillus* sp. (Sigma). Based on A_{340}, the reaction went to 55% completion in 90 min at 21°C. This incubation was followed by a second incubation with 10 mM acetone and 10 units of ADH from *Thermoanaerobium brockii* (Sigma). Based on A_{340}, the reaction went to *ca.* 100% completion in 15 min at 21°C. The resulting [4-^{3}H]$NADP^+$ was then purified by Sephadex G-75 (Pharmacia). Fractions containing the highest activity were combined, concentrated by freeze drying, and further purified by Sephadex G-25 as described in the Results. Fractions containing the highest activity were again combined and concentrated by freeze drying. Stereospecifically reduced nicotinamide coenzymes were generated just prior to PCR analyses as follows: incubation for 40 min of either: (1) 1 mM [4-^{3}H]$NADP^+$ (18 Ci mol^{-1}) with 2 mM unlabeled glucose plus 1 unit GDH; or (2) 1 mM unlabeled $NADP^+$ with 2 mM D-[1-^{3}H]glucose (10 Ci mol^{-1}) plus 1 unit GDH, yielded either (1) [4R-^{3}H]NADPH or (2) [4S-^{3}H]NADPH, respectively.

Enzyme isolation, incubation, and analysis procedures. Etiolated wheat (*Triticum aestivum*) was grown in the dark in moist vermiculite for 8-10 days. Cut seedlings were incubated overnight in 10 mM 5-aminolevulinic acid in 2 mM potassium phosphate, pH 6.8, and Pchlide was isolated from them by the method of Griffiths (4), except that extraction was accomplished by grinding in a mortar with a suspension of 1 mg mL^{-1} $MgCO_3$ in acetone. Etioplasts were prepared in hypertonic sucrose (Buffer I) from freshly cut seedlings by the method of Griffiths (3). Isolated etioplasts were osmotically lysed in hypotonic sucrose (Buffer II) by the method of Beer & Griffiths (6). Etioplast membranes were isolated by centrifugation at 91000*g* for 20 min at 4°C and resuspended for 30 min at 4°C in Buffer I plus 4 mM CHAPS and 15% (v/v) glycerol. The suspension was then centrifuged at 31000*g* for 20 min at 4°C and the supernatant used as a source for the solubilized PCR (7). Incubations were carried out in a final volume of 1.0 mL (containing 100 nmol of either 2R or 2S stereospecifically reduced, tritium labeled NADPH; 2.5 nmol Pchlide; and 0.2 mg solubilized membrane protein) at 21°C with 5 light flashes administered one every minute with a Honeywell Strobonar 202 flasher (or for an identical time in the dark). The incubation mixtures were then freeze dried and extracted 3 times with 2 mL of methanol. The combined methanol extracts were diluted 1:1 with water and extracted 3 times with 4 mL of hexane. The hexane washes were discarded and the aqueous methanol extracts were then extracted 3 times with 5 mL of diethyl ether. The combined ether extracts were washed 3 times with 10 mL water, dried over anhydrous Na_2SO_4, and concentrated to 2 mL with a stream of nitrogen gas. The concentrations of both Pchlide and Chlide were determined spectrophotometrically and the entire extract was counted in 7 mL of Scinti-Verse II (Fisher) with an LKB-Wallac model 1217 RackBeta liquid scintillation counter which had been calibrated for counting efficiencies of between 3.5-40% in the presence of various concentrations of magnesium protoporphyrin monomethyl ester obtained as previously described (8).

RESULTS AND DISCUSSION

The preparation of the 4R and 4S tritium labeled isomers of both NADH and NADPH is summarized in Fig. 1. Use was made of the GDH from *Bacillus* sp., which is specific for the pro S hydrogen of either NADH or NADPH (1). Thus, in the case of $NADP^+$, reduction with D-[1-^{3}H]glucose yields [4S-^{3}H]NADPH. The reaction has an equilibrium constant of approximately 1, but the reaction

GENERATION OF STEREOSPECIFICALLY LABELED NAD(P)H

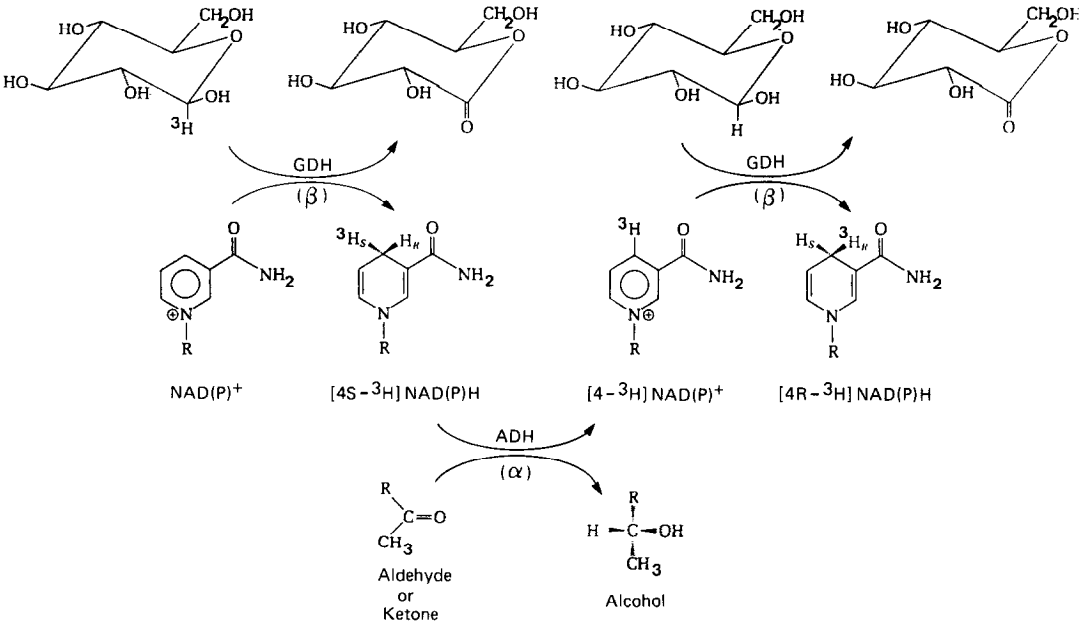

Fig. 1. Scheme for the preparation of stereospecifically (4S and 4R) tritium labeled NAD(P)H. The enzymes, glucose dehydrogenase (GDH) and alcohol dehydrogenase (ADH), are specific for the pro S (or β) and pro R (or α) hydrogens, respectively, of either NADH or NADPH.

usually proceeds to between 55-70% of completion in 1.5 h due, probably, to a nonenzymatic hydrolysis of D-glucono-5-lactone. This incubation was then followed by a second incubation with acetone and the ADH from *Thermoanaerobium brockii*, which is specific for the pro R hydrogen of either NADH or NADPH (1), and has a broad specificity for alcohols. Since the GDH remained active, both enzymes should have continued to cycle until nearly all of the [1-^{3}H]glucose was used up. The resulting [4-^{3}H]NADP$^+$ was then purified by successive passes through Sephadex G-75, in which the enzymes were eluted in the void volume and the NADP$^+$ in the bed volume (Fig. 2), and Sephadex G-25, in which the labeled NADP$^+$ was eluted near the void volume and labeled glucose was eluted somewhat before the bed volume (Fig. 3). The results indicated that very little labeled glucose remained in the sample of [4-^{3}H]NADP$^+$ prepared. Any labeled isopropanol produced by continued enzymatic cycling would have been removed during the freeze drying of the sample between the two gel filtrations. The same reaction sequence may be used to synthesize [4-^{3}H]NAD$^+$. The final specific activity of the [4-^{3}H]NADP$^+$ was found to be 18 Ci mol^{-1}; however, since D-[1-^{3}H]glucose is available in specific activities of between 15-30 Ci mmol^{-1}, much higher specific activities of the labeled NADP$^+$ (or NAD$^+$) can be

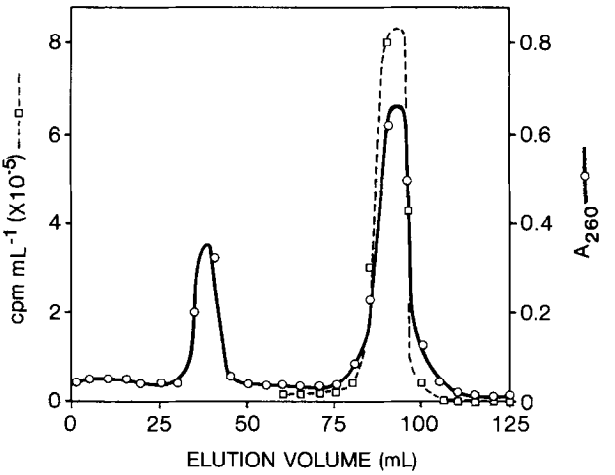

Fig. 2. Gel filtration on Sephadex G-75 (bed volume 100 mL) of [4-^{3}H]NADP$^+$ prepared as described in the Text. Fractions were analyzed for radioactivity (□) and A_{260} (○).

obtained. Finally, [4R-^{3}H]NADPH was generated just prior to use in PCR analyses with GDH and [4-^{3}H]NADP$^+$ plus unlabeled D-glucose, while the corresponding 4S isomer was generated from unlabeled NADP$^+$ plus D-[1-^{3}H]glucose.

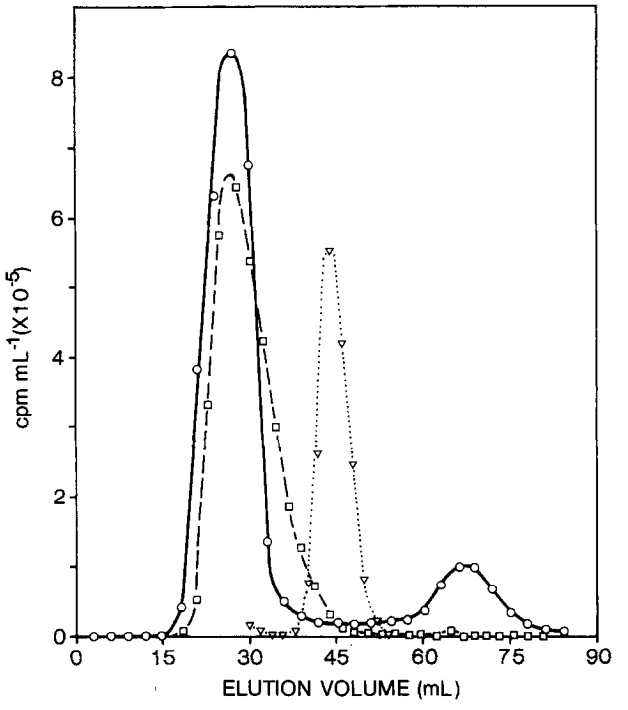

Fig. 3. Gel filtration on Sephadex G-25 (bed volume 65 mL) of (A) [4-^{3}H]NADP$^+$ (□) prepared as described in the Text (and after the gel filtration of Fig. 2); (B) commercial [4-^{3}H]NAD$^+$ (○); and (C) commercial D-[1-^{3}H]glucose (▽). Fractions were analyzed for radioactivity only.

Table I. Nicotinamide Coenzyme Stereospecificity of the NADPH: Protochlorophyllide Oxidoreductase of Etiolated Wheat

Reduced nicotinamide coenzyme	Light/Dark	Extracted radio-activity (nCi)	Extracted Chlide (nmol)	Estimated specific activity of newly formed Chlide (Ci mol^{-1})	(% max)
[4R-^{3}H]NADPH[a]	Light	0.76	0.81	----	---
"	Dark	0.37	0.11	----	---
"	Light - Dark	0.39	0.70	0.56	3.1%
[4S-^{3}H]NADPH[b]	Light	5.38	0.90	----	---
"	Dark	0.21	0.18	----	---
"	Light - Dark	5.17	0.72	7.18	71.8%

[a]Specific activity 18 Ci mol^{-1}
[b]Specific activity 10 Ci mol^{-1}

The results of the incubation of etiolated wheat extracts with Pchlide and either the 4R or 4S stereoisomer of [4-^{3}H]NADPH are shown in Table I. The administration of light flashes during the incubation allowed the conversion of some of the Pchlide to Chlide: in both cases, approx. 0.7 nmol of Chlide in excess of that found in the dark control was present in the extracts of the light-treated samples (Table I) and the molar ratio of Chlide:Pchlide had increased 8-9 fold in the latter (data not shown). However, the estimated specific activity of this newly formed Chlide was much higher for the 4S isomer of [4-^{3}H]NADPH than for the 4R isomer; when expressed as percentages of the theoretical maximum, the values were 72% and 3%, respectively (Table I). Failure to yield Chlide with the maximum theoretical specific activity may have been due to the presence of photoreactive complexes of PCR with endogenous Pchlide and (unlabeled) NADPH in the extracts. Consistent with previous work (8) using magnesium 2,4-divinylpheoporphyrin a_5 as a substrate, we have concluded that the PCR of etiolated wheat is specific for the pro S hydrogen (or B-face) of NADPH.

During a study of the conversion of Pchlide to Chlide, Griffiths (9) had previously used chemically reduced (and hence achiral) [4-^{3}H]NADPH; he was, therefore, unable to investigate the stereospecificity of PCR towards hydride removal from NADPH. However, since an exhaustive flash illumination was carried out in order to deplete endogenous Pchlide and NADPH prior to the addition of exogeous substrates, Griffiths (9) obtained a stoichiometric incorporation of radioactivity from labeled NADPH into Chlide. Recently, Griffiths and Walker (10) have presented evidence that PCR may be a flavoprotein containing FAD. If a hydride is indeed first transfered to a bound flavin, both our results and those of Griffiths (9) imply that the same hydride must subsequently be transfered to the Pchlide. Such a mechanism for the photoconversion of Pchlide to Chlide would require the involvement of a number of transient intermediates. In this respect, a non-fluorescent

transient intermediate absorbing maximally at 690 nm (11) and several other new transient intermediates (12) have been detected during studies of the photoconversion. As yet, however, these intermediates have not been characterized in terms of molecular entities.

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