Methods for the Enzymatic Synthesis of Tyrosine and Phenylalanine Labeled with Nitrogen-13

ALAN S. GELBARD,* ARTHUR J. L. COOPER, YASUHISA ASANO, EDWARD NIEVES,† SABINA FILC-DERICCO and KAREN C. ROSENSPIRE

1Biophysics Laboratory, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021, 2Departments of Biochemistry and Neurology, Cornell University Medical College, New York, NY 10021, U.S.A. and 3Sagami Chemical Research Center, Sagamihara, Kanagawa 229, Japan

(Received 8 February 1989)

L-[13N]Tyrosine and L-[13N]phenylalanine were synthesized using immobilized enzymes by two methods. In method 1, [13N]ammonia is converted to L-[13N]glutamate; transamination with p-hydroxyphenylpyruvate yields L-[13N]tyrosine. [13N]Tyrosine is separated from other labeled intermediates on a Poropak Q column. In method 2, phenylalanine dehydrogenase catalyzes the reversible reductive [13N]amination of either phenylpyruvate or p-hydroxyphenylpyruvate to form L-[13N]phenylalanine or L-[13N]tyrosine, respectively. The feasibility of labeling DOPA and tryptophan with 13N was also demonstrated.

Introduction

The aromatic amino acids, tyrosine and phenylalanine, are of biomedical interest. They are the precursors of some excitatory neurotransmitters, their aberrant metabolism is associated with certain neoplasms and they have been used to measure the rate of protein synthesis in tumors and normal organs such as muscle and liver, as described and reviewed by Hasselgren et al. (1988). Thus, tyrosine and phenylalanine labeled with a positron-emitting radioisotope would be useful for positron emission tomography (PET) studies of tissues and tumors.

L-[1[13C]Tyrosine has been prepared from 11CO2 by the lithium isocyanide method followed by isomeric resolution using HPLC (Bolster et al., 1986). L-[18F]Fluorotyrosine has been prepared by electrophilic radiofluorination with hypo[18F]fluorite and resolution of the D- and L-isomers by an HPLC method based on ion-pairing with dodecylsulfate (Coenen et al., 1988).

An alternative to labeling aromatic amino acids with 13C or 18F is the introduction of the 15N nuclide into the amino position by use of immobilized enzymes. Enzymatic labeling provides a rapid method for synthesizing the biologically active isomer. The short half-life of 15N (9.96 min) permits sequential studies on the same subject. The present communication describes two methods for the enzymatic synthesis of either L-[13N]tyrosine or L-[13N]phenylalanine.

The first method involves the glutamate dehydrogenase-catalyzed conversion of [15N]ammonia to L-[15N]glutamate; transamination with p-hydroxyphenylpyruvate yields L-[15N]tyrosine. Transfer of the amino group from L-[15N]glutamate to p-hydroxyphenylpyruvate is catalyzed by the aspartate aminotransferase preparation (which has some inherent tyrosine aminotransferase activity). Labeled tyrosine is separated from the other labeled intermediates of these reactions by use of a Poropak Q column (Niederwieser, 1971). Both enzymes used in the synthesis are readily available from commercial sources.

The second method utilizes phenylalanine dehy-
Materials and Methods

Beef liver glutamate dehydrogenase with a specific activity of 120 U/mg. and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Boehringer Mannheim (Indianapolis, Ind.). Pig heart cytosolic aspartate aminotransferase, 500 U/mg, was purchased from Calbiochem (San Diego, Calif.). Phenylalanine dehydrogenase, 179 U/mg, was purified by anion-exchange and gel filtration column chromatography on an Escherichia coli transformant harboring the gene for the Bacillus sphaericus enzyme (Asano et al., 1987a). p-Hydroxyphenylpyruvate acid, dihydroxyphenylalanine and L-DOPA were purchased from Sigma Chemical Co. (St Louis, Mo). CNBr-activated Sepharose was purchased from Pharmacia (Piscataway, N.J.). Poropak Q, with a mesh size of 100-200 was purchased from Supelco Inc. (Bellefonte, Pa). glucamate dehydrogenase with a specific activity of 120 U/mg. and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Boehringer Mannheim (Indianapolis, Ind.). Pig heart cytosolic aspartate aminotransferase, 500 U/mg, was purchased from Calbiochem (San Diego, Calif.). Phenylalanine dehydrogenase, 179 U/mg, was purified by anion-exchange and gel filtration column chromatography on an Escherichia coli transformant harboring the gene for the Bacillus sphaericus enzyme (Asano et al., 1987a). p-Hydroxyphenylpyruvate acid, dihydroxyphenylalanine and L-DOPA were purchased from Sigma Chemical Co. (St Louis, Mo). CNBr-activated Sepharose was purchased from Pharmacia (Piscataway, N.J.). Poropak Q, with a mesh size of 100-200 was purchased from Supelco Inc. (Bellefonte, Pa).

CNBr-activated Sepharose was purchased from Pharmacia (Piscataway, N.J.). Poropak Q, with a mesh size of 100-200 was obtained from Supelco Inc. (Bellefonte, Pa).

\[^{15}\text{N}\text{Ammonia was produced in a CS-15 cyclotron (Cyclotron Corp., Berkeley, Calif.) by the (p, n) reaction on water and subsequent reduction of}^{15}\text{Nitrate and nitrite with DeVarda’s alloy and sodium hydroxide (Gelbard et al., 1975). Typical yields of}^{15}\text{N-ammonia were 150-300 mCi in a final volume of 3 mL with a sp. act. ca. 1 Ci/μmol.}

Glutamate dehydrogenase was immobilized onto 0.5 g CNBr-activated Sepharose as previously described (Gelbard et al., 1980). Aspartate aminotransferase (5000 IU) was immobilized onto 2 g CNBr-activated Sepharose in a similar manner by mixing the enzyme and the support material with 10 μmol p-hydroxyphenylpyruvate and 4 μmol NADH in 13 mL of 50 mM sodium phosphate buffer (pH 8.0) and shaking the slurry overnight at 4 °C with a wrist action shaker (Burrell Corp., Pittsburgh, Pa). The suspension was poured onto a glass column and the unbound enzyme was removed by washing the column with 500 mL of 50 mM phosphate buffer (pH 8.0). phenylalanine dehydrogenase (250 IU) was immobilized onto 1 g CNBr-activated Sepharose by dialyzing the enzyme against 50 mM sodium phosphate buffer (pH 7.0) overnight and shaking the enzyme and the support material with 10 μmol p-hydroxyphenylpyruvate and 4 μmol NADH in 15 mL of 50 mM sodium phosphate buffer (pH 7.0) for 4 h at 4 °C. The column was washed free of unbound protein with 500 mL of 50 mM sodium phosphate buffer (pH 8.0).

In experiments involving the catalytic exchange between L-DOPA and \[^{15}\text{N-ammonia, 75 IU of soluble phenylalanine dehydrogenase was added to a reaction mixture (3.5 mL) of 50 mM sodium phosphate buffer (pH 6.2) containing 30 μmol L-DOPA, 3 μmol NADH and 300 mCi \[^{15}\text{N-ammonia. The solution was kept under an atmosphere of helium and was protected from light for the 10 min incubation period at 37 °C.}}

A 10 × 2 cm column bed of Poropak Q was prepared as follows. Powdered Poropak Q (10 g) was added to 50 mL of acetone and allowed to swell for 1 h. The slurry was washed 8 times with 50 mL of water and then washed twice with 50 mL of 150 mM sodium phosphate buffer (pH 2.0). The slurry was poured into a glass column and the column was washed twice with the acidic phosphate buffer.

The procedure for synthesizing L-[\(^{15}\text{N}\)]-tyrosine by coupling the glutamate dehydrogenase and the aminotransferase reactions was as follows. \[^{15}\text{N-ammonia was collected in 3 mL of 50 mM sodium phosphate buffer (pH 8.0) and was added to the combined eluates from the glutamate dehydrogenase column (which contains L-[\(^{15}\text{N}\)]glutamate and unreacted \[^{15}\text{N-ammonia). This solution (6 mL) was then transferred onto the 2 g CNBr Sepharose column containing aspartate aminotransferase. The reaction mixture was incubated on the column for 10 min at room temperature. At the end of this time, the mixture was forced through the column under air pressure and the column was washed with 3 mL of 50 mM sodium phosphate buffer (pH 8.0). To the combined eluates was added 200 μL of 5 N HCl and this solution was placed on a 10 × 2 cm column bed of Poropak Q. The Poropak Q column was washed with 10 mL of 50 mM sodium phosphate buffer (pH 2.0). The column was subsequently washed twice with 6 mL of 50 mM sodium phosphate buffer, (pH 12.0). The last wash which contained the labeled tyrosine was neutralized by the addition of 100 μL of 5 N HCl, made isotonic with sodium chloride, and was passed through a 0.22 μm millipore filter.

In experiments to determine the ability of the immobilized preparation of aspartate aminotransferase to catalyze the transfer of the \(^{15}\text{N-labeled amino group from L-[\(^{15}\text{N}\)]glutamate to form L-[\(^{15}\text{N}\)]-phenylalanine or L-[\(^{15}\text{N}\)]-tryptophan, a suspension of 100 μmol of phenylpyruvate or indolyl-3-pyruvate, respectively, replaced p-hydroxyphenylpyruvate.
The synthesis of L-[\textsuperscript{15}N]phenylalanine or L-\textsuperscript{15}N]tyrosine by the phenylalanine dehydrogenase catalyzed reductive \textsuperscript{15}N amination of the appropriate precursor was carried out in the following manner: 150–250 mCi of \textsuperscript{15}N ammonia was collected in 3 mL of 50 mM sodium phosphate buffer (pH 8.0); 20 pmol of solid sodium phenylpyruvate and 2 \textmu mol of NADH were added to the solution which was then allowed to run onto the Sepharose column containing the immobilized enzyme. After incubation at room temperature for 5 min the column was then washed with 3 mL of 50 mM sodium phosphate buffer (pH 8.0). The combined solutions were passed through an AG 30 cation-exchange column that had been adjusted to pH 8.0 with 50 mM sodium phosphate buffer. Unreacted \textsuperscript{15}N ammonia was trapped on the cation-exchange column while the \textsuperscript{15}N-labeled amino acid passed through. The column was then washed twice with 3 mL of the phosphate buffer to recover additional labeled amino acid.

Analyses of the reaction mixtures and the purified \textsuperscript{15}N-labeled compounds were carried out by HPLC. The analytical system consisted of a Perkin-Elmer Series 4 in which the effluent was continuously monitored with a Ramona D-flow-through \gamma-detector (IN/US Service Corp., Fairfield, N.J.) with data processing capability for integrating peaks and correcting for background and natural decay (for further details of this system, see Nieves et al. [1986]). A Partisil 10 SAX (Phenomenex, Rancho Palo Verdes, Calif.) analytical anion-exchange column (10 \mu m, 4.6 \times 250 mm) was used for the separation of labeled compounds. The eluting buffer was 5 mM potassium phosphate (pH 3.5).

To further characterize the purified labeled amino acid, 20-\mu L samples were injected onto a Partisil SCX analytical cation-exchange column (10 \mu m, 4.6 \times 250 mm) and eluted with 5 mM potassium phosphate-phosphoric acid (pH 7.5) for 8 min followed by 20 mM potassium phosphate-phosphoric acid (pH 3.5) for a further 12 min; at 25°C (Cooper et al., 1987).

Results

The coupled enzymatic reactions of glutamate dehydrogenase and aspartate aminotransferase with \textit{p}-hydroxyphenylpyruvate as the amino acceptor resulted in efficient conversion of \textsuperscript{\textit{15}N} ammonia to L-\textsuperscript{\textit{15}N]tyrosine via labeled glutamate. Figure 1(A) shows an analysis of the reaction mixture after passage through a column containing immobilized glutamate dehydrogenase and a subsequent 10 min incubation on the column containing immobilized aspartate aminotransferase. The \textsuperscript{15}N-labeled components in the mixture are ammonia, tyrosine and glutamate. Approximately 60% of the total \textsuperscript{15}N activity is in labeled tyrosine. After passage of the reaction mixture through and subsequent acid wash of the Poropak Q column, the two predominant labeled components are ammonia and glutamate [Fig. 1(B)]. The Poropak Q column is then washed twice with 3 mL of 50 mM sodium phosphate buffer (pH 12.0). HPLC analysis showed that the second wash contained 98% radiochemically pure L-\textsuperscript{\textit{15}N]tyrosine [Fig. 1(C)]. The total time for synthesis of L-\textsuperscript{\textit{15}N]tyrosine by this procedure is 20 min from the time of collection of \textsuperscript{\textit{15}N} ammonia; 20 mCi of labeled tyrosine can be prepared from 175-250 mCi of \textsuperscript{\textit{15}N} ammonia.

The ability of the aspartate aminotransferase preparation to catalyze the transfer of the labeled amino group of glutamate to the precursors of phenylalanine and tryptophan was determined next. With phenylpyruvate or indolyl-5-pyruvate as the amino acceptors in place of \textit{p}-hydroxyphenylpyruvate, approx. 30 or 15% of total \textsuperscript{15}N activity was converted to L-\textsuperscript{\textit{15}N]phenylalanine or L-\textsuperscript{\textit{15}N]tryptophan, respectively. No attempt was made to purify these amino acids from labeled ammonia and glutamate.

Phenylalanine dehydrogenase has the ability to catalyze the reductive \textsuperscript{15}N amination of the respective \textit{\alpha}-keto acids of phenylalanine and tyrosine with high efficiency. After 5 min incubation of \textsuperscript{\textit{15}N} ammonia and either phenylpyruvate or \textit{p}-hydroxyphenylpyruvate with the enzyme immobilized on the CNBr-activated Sepharose column 83% of the \textsuperscript{\textit{15}N} ammonia was converted to L-\textsuperscript{\textit{15}N]phenylalanine and, in a separate experiment, 38% of the \textsuperscript{15}N was converted to L-\textsuperscript{\textit{15}N]tyrosine, respectively. The labeled amino acids were purified by passage of
the solution through an AG-50 column equilibrated with 50 mM sodium phosphate buffer (pH 8.0). Yields of each labeled amino acid were >30 mCi. The identities of purified L-[13N]phenylalanine and L-[15N]tyrosine were confirmed by comparison of their retention times to those of unlabeled authentic standards on SCX columns (tyrosine = 19.5 min; phenylalanine = 7.0 min).

L-DOPA was labeled by the exchange reaction between L-DOPA and [15N]ammonia in the presence of NAD+ and phenylalanine dehydrogenase; 9% of the label was transferred to L-DOPA. In a separate experiment, involving the exchange reaction between L-phenylalanine and [15N]ammonia, transfer of label from [15N]ammonia to phenylalanine was 50%. It should be pointed out that exchange reactions for labeling, unlike enzymatic syntheses, result in products with low specific activities.

**Discussion**

Compounds labeled with positron-emitting radionuclides are used to measure specific metabolic functions in the whole animal by means of PET. The possibility of using amino acids with positron-emitting isotopes for the in-vivo measurement of protein synthesis has been discussed. L-Methyl-[^14C]methionine has been suggested as a marker for cerebral protein synthesis (Bustany and Comar, 1985). However, other studies have shown that following administration of L-[1-14C]methionine or L-methyl[^13C]methionine, labeled metabolites are found in protein-free fractions of brain, liver, pancreas and Walker 256 carcinosarcoma. Appreciable formation of labeled metabolites complicates the kinetic model for a quantitative in-vivo measurement of the protein synthetic rates with PET (Ishiwata et al., 1988b). A compartmental model approach for estimation of human cerebral protein synthesis with L-[1-14C]leucine as the labeled amino acid has been described (Keen et al., 1987). Smith et al. (1988) noted, that in the brain and liver of adult rats there is significant recycling of leucine derived from protein degradation into the precursor pool for protein synthesis and the resulting dilution of specific activity must be taken into account. L-[1-14C]Tyrosine and L-[2-15F]fluorotyrosine have been proposed as markers for determining the rate of protein synthesis in tissues (Ishiwata et al., 1988a; Coenen et al., 1988). In the present report, we describe two methods for the synthesis of L-tyrosine and L-phenylalanine labeled with 15N. Because of the ease with which these aromatic amino acids can be labeled with 15N, they may serve as an alternative to L-[1-14C]tyrosine or L-[2-15F]fluorotyrosine for studying the rate of protein synthesis. Unlike the 15F label, the short half-life of 15N makes it adaptable for repeated studies on the same individual on the same day. L-[15N]Tyrosine may be useful for the study of protein synthesis in those organs with little tyrosine aminotransferase activity. The degree to which formation of labeled intermediates through either transamination or catabolic reactions occurs in such tissues over a period of 30-60 min must be investigated. In addition to using L-[15N]tyrosine or L-[1-13N]phenylalanine to study protein synthesis, these labeled amino acids may prove useful for studying catecholeamine or melanin metabolism in cases of neuroblastoma or melanoma.

We report two enzymatic procedures for the labeling of L-[15N]tyrosine or L-[1-13N]phenylalanine. The reductive 15N amination of p-hydroxyphenylpyruvate or phenylpyruvate in a reaction catalyzed by phenylalanine dehydrogenase produces either L-[15N]tyrosine or L-[1-13N]phenylalanine in high yields that is easily purified from unreacted [15N]ammonia by passage through a cation-exchange column. Thus, this method is the procedure of choice for preparing L-[15N]tyrosine and L-[1-13N]phenylalanine. Phenylalanine dehydrogenase, however, was not commercially available until recently and the enzyme had to either prepared by the investigator or by custom synthesis.* These procedures are expensive. An alternative is to utilize the coupled glutamate dehydrogenase-aspartate aminotransferase reactions. Both enzymes are available from commercial sources. We therefore report this second procedure and a novel method of separation of tyrosine from other labeled intermediates by means of differential elution from a Poropak Q column. Our previous attempts to rapidly separate labeled tyrosine from glutamate and ammonia by the use of cation- and anion-exchange chromatography or preparative HPLC did not result in a product of high enough radiochemical purity or yield for use in further biological or clinical studies. This problem has now been resolved by the use of a Poropak Q column to separate the labeled intermediates from L-[15N]tyrosine. Finally, we have demonstrated that it may be feasible to label DOPA and tryptophan with 15N.

*Phenylalanine dehydrogenase is now available from Central Glass Company Ltd, 3-7-1 Kandamiishiki-cho, Chiyoda-ku, Tokyo 101, Japan.

**References**

Synthesis of $^{13}$N-labeled aromatic amino acids

phenylalanine dehydrogenase produced in E. coli—its purification and application to L-phenylalanine synthesis. 
Agric. Biol. Chem. 51, 2621.
Niederwieser A. (1971) Use of neutral polystyrene resin for rapid desalting and fractionation of non-polar amino acids and non-polar oligopeptides. J. Chromatogr. 61, 8.