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Note

Assay for trimethyllysine hydroxylase by high-performance liquid chromatography

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The first enzymatic step in the carnitine biosynthetic pathway is the hydroxylation of 6-N-trimethyllysine to form L-*erythro*-3-hydroxy-6-N-trimethyllysine via the mitochondrial enzyme trimethyllysine hydroxylase [1, 2]. Carnitine is required for the mitochondrial β-oxidation of long-chain fatty acids. Carnitine has also been proposed to have numerous other functions, such as the detoxification of excess acyl coenzyme A (CoA) compounds, modulation of the acyl CoA/free CoA ratio, and shuttling of acyl compounds between various cellular compartments [3].

Trimethyllysine hydroxylase has been measured previously by radioenzymatic assays utilizing the ^{14}C- or ^{3}H-labelled substrate, trimethyllysine [1, 4–6]. Assays utilizing non-labelled trimethyllysine have not been developed. However, a report describing a high-performance liquid chromatographic (HPLC) assay for trimethyllysine indicated that 3-hydroxy-6-N-trimethyllysine, the product of trimethyllysine hydroxylase, was separable from trimethyllysine under the conditions of the assay [7].

This paper describes a new assay for trimethyllysine hydroxylase. The assay does not require the use of radiolabelled trimethyllysine and utilizes an internal standard, triethyllysine.

EXPERIMENTAL

Reagents

Acetonitrile (HPLC, UV grade) was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Mercaptoethanol and the σ-phthalicdicarboxaldehyde

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(sold as fluoraldehyde) were obtained from Pierce (Rockford, IL, U.S.A.). The ion-exchange resins Dowex 50W-X8 (200–400 mesh, H^+) and Dowex 1-X8 (200–400 mesh, Cl^-) were purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Trimethyllysine and triethyllysine were prepared as described previously [8]. The sample of L-*erythro*-3-hydroxy-6-N-trimethyllysine used to standardize the assay was a gift from Dr. C.L. Hoppel (Cleveland, OH, U.S.A.). All other reagents were of analytical-reagent grade from commercial sources.

Sample preparation

Male Sprague–Dawley rats (Charles River, Portage, MI, U.S.A.), 175–225 g, were fed for one week a trimethyllysine-limiting diet, with 7.9 nmol trimethyllysine per g diet [9]. The animals were decapitated, four fed animals and four animals after two days of starvation. Kidneys were removed and quickly frozen via freeze clamps cooled in liquid nitrogen. The tissues were then homogenized the same day in 200 m*M* mannitol, 70 m*M* sucrose and 5 m*M* 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 7.4 (1 g tissue per 3 ml buffer). The homogenate was assayed directly for trimethyllysine hydroxylase activity. Homogenate protein concentrations were measured by the method of Lowry et al. [10].

Assay

The conditions for the enzyme assay were a modification of the trimethyllysine hydroxylase assay reported by Sachan and Hoppel [4]. The incubation medium was 75 m*M* Tris–HCl (pH 8.0), 2.5 m*M* ascorbate, 5.0 m*M* α-ketoglutarate, 0.5 m*M* dithiothreitol, 0.5 m*M* iron(II) sulfate and 5.0 m*M* calcium chloride. The medium, in 65 μl, was incubated with 75 μl of the tissue homogenate for 5 min in a shaking water-bath at 37°C. The reaction was initiated by the addition of 50 μl of 20 m*M* trimethyllysine for the kidney assay. The assay was analyzed in duplicate, while a blank was also analyzed. The assays were terminated after 3 min by the addition of 500 μl of 95% ethanol. The reaction times were within the range described by Sachan and Hoppel [4], and the reaction rates were shown to be linear under the conditions used. After the ethanol addition, trimethyllysine was added to each of the blanks, and triethyllysine was added to all of the samples. The compounds of interest from the deproteinized sample were obtained via ion-exchange–ion-exclusion chromatography as described previously [7]. Standard curves were prepared by passing a standard solution of 3-hydroxy-6-N-trimethyllysine and triethyllysine over the ion-exchange–ion-exclusion columns and processing as for the tissue samples. Standard concentrations of 3-hydroxy-6-N-trimethyllysine ranged between 10 and 60 nmol/ml. The eluates were evaporated in a centrifugal evaporator (Speed Vac concentrator Model 100H, Savant Instruments, Hicksville, NY, U.S.A.) and reconstituted in 200 μl of distilled water. A 50-μl aliquot of sample was then mixed with 50 μl of an *o*-phthalicdicarboxaldehyde mixture (50 mg of *o*-phthalicdicarboxaldehyde, 1.25 ml of methanol, 11.25 ml of 0.4 *M* sodium borate, pH 9.5, and 50 μl mercaptoethanol) and mixed for 1 min. A 100-μl aliquot of 0.1 *M* sodium acetate, pH 6.4, was then added, and the entire mixture injected onto the chromatographic system. Differences between

enzyme activities were determined by the unpaired t-test [11]. Significant differences were determined at $p < 0.05$.

Chromatography

The HPLC system consisted of two Model 110A pumps, a Model 421 controller, a Model 210 injector, and an Altex 3-μm Ultrasphere ODS (7.5 cm $\times$ 4.6 mm I.D.) column (Beckman Instruments, Fullerton, CA, U.S.A.), a Shimadzu C-R1A data processor (Shimadzu, Kyoto, Japan), and Gilson Spectra Glo Filter fluorometer (Gilson Medical Electronics, Middleton, WI, U.S.A.). The two mobile phases were 0.1 *M* sodium acetate, pH 6.4 (reagent A) and acetonitrile (reagent B). The gradient used for the assay is as follows: 0–6 min, linear increase from 6 to 10% acetonitrile; 10–14 min, linear increase from 10 to 20% acetonitrile; 14–23 min, linear increase from 20 to 22% acetonitrile; 23–23.5 min, linear increase from 22 to 80% acetonitrile; 26.5–27 min, linear decrease from 80 to 6% acetonitrile. The column was re-equilibrated for 8 min prior to the next injection. The flow-rate was 1.7 ml/min. The excitation filter was 7-60X (330–380 nm), while the emission filter was 3-73M (430–600 nm).

RESULTS AND DISCUSSION

The chromatographic profiles showing 3-hydroxy-6-N-trimethyllysine, trimethyllysine, and triethyllysine, are shown in Fig. 1. Fig. 1A shows a blank from rat kidney for the reaction, in which trimethyllysine was added after the addition of 95% ethanol. Peak 2 is triethyllysine, while the large peak (retention time 18.4 min) is trimethyllysine. Fig. 1B shows a chromatogram from a trimethyllysine hydroxylase reaction from rat kidney, in which peak 1 is 3-hydroxy-6-N-trimethyllysine. The product was identified by co-chromatographing with a previously obtained sample of L-*erythro*-3-hydroxy-6-N-trimethyllysine.

Standard curves obtained on four successive days showed a linear relationship between concentration of 3-hydroxy-6-N-trimethyllysine (nmol/ml) and the height ratio of 3-hydroxy-6-N-trimethyllysine to triethyllysine, with a mean r^2 for four analyses of 0.993. Slopes of these four replicate standard curves had a relative standard deviation (R.S.D.) of 9.8%. The R.S.D. of three replicate determinations of a rat kidney was 5.7%. The R.S.D. for three successive days from two rat kidneys, in terms of enzyme activity, was 6.1 and 5.4%, respectively.

The renal enzyme activities for fed and starved animals were 0.400 ± 0.040 and 1.084 ± 0.049 nmol 3-hydroxy-6-N-trimethyllysine formed per mg protein per min, respectively (mean $\pm$ standard error of the mean). Kidneys from starved rats showed significantly higher trimethyllysine hydroxylase activity than kidneys from fed rats. The values for enzyme activity for kidney in the fed rat compare to a range of 0.12–5 nmol 3-hydroxy-6-N-trimethyllysine per mg protein per min as reported in the literature [1, 4–6].

The HPLC trimethyllysine hydroxylase assay described herein requires approximately 35 min between samples, with 27 min to run the assay and an additional 8 min to re-equilibrate the column. The assay has the advantage of not

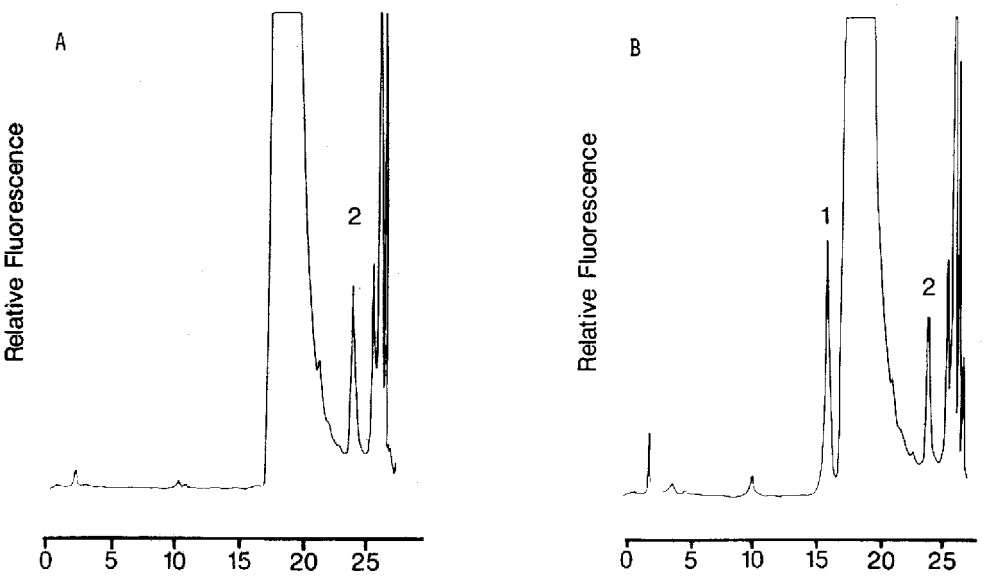

Fig. 1. Chromatogram of renal trimethyllysine hydroxylase assay. (A) Blank, i.e., trimethyllysine added after deproteinization of the enzyme; (B) trimethyllysine added at the start of the reaction. Peak 1 is 3-hydroxy-L-*erythro*-trimethyllysine and peak 2 is triethyllysine. Chromatographic conditions: 3-μm Ultrasphere ODS column (75 × 4.6 mm I.D.); gradient elution, 0.1 *M* sodium acetate, pH 6.4, and acetonitrile (gradient described in text); flow-rate, 1.7 ml/min. The fluorometric detector was operated with an excitation filter of 330–380 nm and an emission filter of 430–600 nm.

requiring a labelled substrate. The assay also uses an internal standard to aid in the accurate quantification of L-*erythro*-3-hydroxy-6-N-trimethyllysine.

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