SYNTHESIS OF OXYGEN-17 LABELED THYROTROPIN RELEASING HORMONE

E. Ponnusamy and D. Fiat

Department of Physiology and Biophysics

University of Illinois at Chicago

Chicago, Illinois 60680

SUMMARY

Oxygen-17 isotope was introduced into the α-carboxyl group of L-proline and L-pyroglutamic acid by acid catalysed exchange of oxygen-17 from $H_2{}^{17}O$ or acid hydrolysis of respective amino acid methyl esters in presence of HCl/ $H_2{}^{17}O$. The α-amino group of the amino acids were protected by tert-butyloxycarbonylation. Two analogues of thyrotropin releasing hormone, one enriched with [^{17}O] at the proline carboxamide oxygen (I) and the other at the pyroglutamic acid's amide oxygen (II) were synthesized using Merrifield method of solid phase peptide synthesis.

Keywords: [^{17}O] labeling, [^{17}O] proline, [^{17}O] pyroglutamic acid, [^{17}O] thyrotropin releasing hormone, solid phase synthesis.

INTRODUCTION

The thyrotropin releasing factor or hormone (TRF or TRH) was isolated in a state of high purity from ovine hypothalami by Guillemin, et al (1), and from porcine hypothalami by Schally, et al (2). Conformational studies of TRH have become of increasing interest in recent years owing to their importance in physiological functions (3-9). Each residue in the TRH molecule plays an important role in determining the conformation of the molecule. The solution conformation of TRH still remains controversial and hence we decided to use [^{17}O] NMR, which has been shown to be an effective tool in studies of the intra- and intermolecular hydrogen bonding, molecular reorientation, bio-molecular structure and dynamics (10-12). The utilization of oxygen magnetic resonance as a probe in the investigation of biologically related processes encounters difficulties due to the low natural abundance of the [^{17}O] isotope (0.037 percent) that possesses a magnetic moment. Its utilization

0362-4803/85/111135-07$01.00

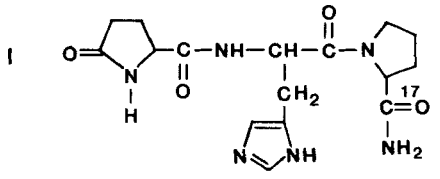
Received April 29, 1985
Revised May 29, 1985

requires enrichment of biomolecules in the [^{17}O] isotope. In the present study we report the synthesis of [^{17}O] isotope labeled TRH (I and II) by solid phase method (13).

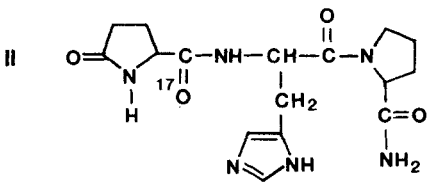
RESULTS AND DISCUSSION

We synthesized oxygen - 17 isotope labeled at the α-carboxyl group of L-proline and L-pyroglutamic acid by acid catalysed exchange of oxygen-17 from $H_2{}^{17}O$ (14) or by acid hydrolysis of respective amino acid methyl esters in the presence of $HCl/H_2{}^{17}O$. In both the mehods, the yields of [^{17}O] enrichment was comparable. These amino acids labeled in both the carboxylic oxygens were prepared with 16 and 17 atom percent [^{17}O] (measured by [^{17}O] NMR) for proline and pyroglutamic acid respectively. The α-amino group of the amino acids were protected by tert-butyloxycarbonylation (15) and the [^{17}O] enrichment was conserved during this critical step.

Since TRH has a carboxamide terminal group, a benzhydrylamine resin (16,17) was employed as a polymer support to synthesize [^{17}O] labeled TRH using BOC-amino acids. In the case of ([^{17}O] pro) TRH (I), the coupling of [^{17}O] BOC-Pro was repeated 3 times with 216 mg (1 mmole) of [^{17}O] BOC-Pro and equivalent amounts of dicyclohexylcarbodiimide (DCCI) in CH_2Cl_2, each 4



II



hrs. In the case of ([^{17}O] p-Glu) TRH (II), the final amino acid, [^{17}O] p-Glu was coupled three times with 230 mg (1 mmole) of [^{17}O] p-Glu and equivalent amounts of DCCI in DMF, each 3 hrs. Since [^{17}O] enriched amino acids are very expensive, we did three repeated couplings with minimum amounts of [^{17}O] enriched materials. The N^{im} of histidine was unprotected and the pyroglutamic acid was used without any protection for peptide synthesis. The final peptide was cleaved from the resin by HF and purified by column chromatography. Standard synthetic TRH supplied by Peninsula Laboratories, Inc. and TRH (unlabeled) synthesized by us were identical with I and II as ascertained by tlc (see experimental) and amino acid analysis. Using mass spectrometry at 40 eV, a molecular ion was detected with m/e 363 confirming the presence of oxygen-17 isotope in I and II. Further investigations on the solution conformation of TRH utilizing I and II by [^{17}O] NMR are in progress.

EXPERIMENTAL

A Schwarz/Mann Peptide Synthesizer was employed for TRH synthesis. Chemical ionization mass spectra were run on a Finnigan MAT 4510 GC/MS at 40 eV. [^{17}O] enrichment was measured on a Bruker CXP-180 high power pulsed Fourier transform spectrometer operating at a frequency of 24.4 MH_z. Peptides were hydrolysed by treatment with constant boiling HCl for 20 hrs at 110^{o}C in vacuo and the amino acid analysis were performed on a Durrum D-500 amino acid analyzer. [^{17}O] water (43.3 percent [^{17}O]) used for labeling was obtained from Monsanto Research Corporation, Miamisburg, OH.

Enriched $H_2{}^{17}O$ was saturated with dry HCl gas and then diluted with equal volume of $H_2{}^{17}O$. Up to 1g/ml amino acid or amino acid methyl ester was dissolved, degased, sealed under vacuum and kept at 105°C for 16-20 hrs (standard conditions of protein acid hydrolysis where racemization and decomposition are minimal) (18). Following removal and quantitative recovery

of $H_2{}^{17}O$ by high vacuum distillation at room temperature, the [^{17}O] amino acid was twice taken up in 5-10 ml water/g amino acid and lyophilized immediately. No side products were detected by amino acid analysis or by tlc when plates were developed with a neutral and a basic or acidic solvents (a. methanol-water-pyridine (20:5:1); b. n-propanol - water (7:3); c. n-propanol-ammonium hydroxide (7:1) and d. n-butanol-acetic acid-water (4:1:1) by volume). The α-amino group was protected by BOC-group (15) and utilized for peptide synthesis.

Benzhydrylamine resin (divinylbenzene 2 percent, 100-200 mesh, 0.2-0.5 mmole of amine/g) (3g) was loaded into the reaction vessel, allowed to swell in CH_2Cl_2 for a few minutes and washed with CH_2Cl_2 (3x30 ml). Further steps in the synthesis of I were as follows: (a) [^{17}O] BOC-L-proline (216 mg, 1 mmole) dissolved in CH_2Cl_2 was added to the reaction vessel and an equimolar amount of DCCI in CH_2Cl_2 was added and the reaction mixture was maintained for 4 hrs. The Resin was washed with CH_2Cl_2 (3x30 ml) and two repeated couplings were carried out with equivalent amounts of [^{17}O] BOC-L-Proline and DCCI in CH_2Cl_2, each 4 hrs. The couplings were monitored by Kaiser's ninhydrin color test (19). The unreacted sites of the resin were then blocked by reaction with 1-acetylimidazole. (b) The BOC-protecting group was removed using 30 ml of trifluoroacetic acid-anisole-CH_2Cl_2 (48:2:50) for 45 minutes. Deprotection was followed by three washings with CH_2Cl_2. (c) Then the resin was neutralized with 20 percent diisopropyle-thylamine in CH_2Cl_2 for 10 minutes followed by washes with CH_2Cl_2 (3x30 ml). (d) BOC-L-Histidine (510 mg, 2 mmole) was attached to the proline bound resin with equivalent amounts of DCCI and 1-hydroxybenzotriazole in DMF (4 hrs) followed by two repeated couplings 2 and 4 hrs. The deprotection and neutralization were carried out the same as in (b) and (c). (e) Neutralization after the second addition was followed by washes with DMF, CH_2Cl_2 and

DMF (3x30 ml). L-Pyroglutamic acid (458 mg, 2 mmole) and equivalent amount of DCCl dissolved in DMF were added to the reaction vessel and the reaction was maintained for 5 hrs and repeated with equivalent amounts of reagents (3 hrs). The resin was then washed with DMF, CH_2Cl_2 and finally with ethyl alcohol (3x30 ml) and dried in vacuo over P_2O_5. (f) The final product (1) was cleaved from the resin with HF as described below: The dried resin peptide was transferred into a kel-F reaction vessel for HF cleavage. Anisole (1 ml/g resin) was added to protect the product. HF was distilled into the reservoir vessel (10 ml/g resin) and then transferred to the reaction vessel. The reaction mixture was stirred for 1 hr at room temperature. The HF was then evaporated from the reaction vessel by water suction. The cleavage mixture was washed several times with ethyl acelate to remove traces of anisole and dried under vacuum. The crude product was then extracted with 1 percent acetic acid and freeze-dried immediately.

The purification of the cleaved peptide was by column chromatography on a 20 x 2.5 cm column packed with 20 g silica gel. The sample was applied as a solution in MeOH-$CHCl_3$, 1:2 ratio, and elution was accomplished with MeOH-$CHCl_3$, 2:1 ratio, 5 ml fractions were pooled (Rf 0.3 on tlc), evaporated to dryness under vacuum and dissolved in water and the resulting solution was lyophilized immediately. The purified compound (48 percent yield) was homogeneous on tlc in five different systems: a. methanol-chloroform (6:3); b. Chloroform-methanol-ammonia (12:9:4); c. 1-butanol-ethyl acetate-acetic acid-water (1:1:1:1); d. chloroform-methanol-30 percent acetic acid (6:4:2) and e. 1-butanol-acetic acid-water (4:1:1). In all these processes, the[^{17}O] enrichment was conserved. Amino acid analysis following 22 hrs hydrolysis gave the following molar ratios: p-Glu, 1.0; His, 1.1; Pro, 1.1; NH_3, 1.65. Similar synthesis was performed to synthesize II utilizing BOC-L-proline, BOC-L Histidine and [^{17}O] L-pyroglutamic acid. The [^{17}O] enrichment in the final products were found to be 14 percent (I) and 16 percent (II) by [^{17}O] NMR.

ACKNOWLEDGEMENT

This work was supported by NIH Grant No. GM24813

REFERENCES

1. Guillemin, R., Sakiz, E. and Ward, D.N. - Proc. Soc. Exp. Biol. med., 118: 1132 (1965).

2. Schally, A.V., Bowers, C.Y., Redding, T.W. and Barrett, J.F.- Biochem. Biophys. Res. Commun., 25: 165 (1966).

3. Locke, W. and Schally, A.V.- The Hypothalamus and Pituitary in Health and Disease, Charles C. Thomas, Springfield, Illinois, 1972.

4. Burgess, A.W., Momany, F.A. and Scheraga, H.A.-Proc. Nat. Acad. Sci. USA, 70: 1456 (1973).

5. Hruby, V.J.- in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, vol. 3, Weinstein, B. Ed., Dekker, New York, p. 1188, 1974.

6. Hughes, J. - Centrally Acting Peptides, University Park Press, Baltimore, Maryland, 1978.

7. Marshall, G.R., Gorin, F.A. and Moore, M.L. -in Annu. Rep. Med. Chem. 13: 227 (1978).

8. Momany, F.A. - in Topics in Current Physics, R.M. Metzger, Ed., Springer-Verlag, Berlin, 1980.

9. Manavalan, P. and Momany, F.A.-Biopolymers, 19: 1943 (1980).

10. Fiat, D., Burgar, M.I., Dhawan, D., St. Amour, T., Steinschneider, A and Valentine, B.-in Neurohypophyseal Peptide Hormones and other Biologically Active Peptides, Schlesinger, D.H. Ed., Elsevier, New York, p. 239, 1981.

11. Burgar, M.I. and Fiat, D. - Period. Biol. 85: 121 (1983).

12. Gilboa, H., Steinschneider, A., Valentine, B., Dhawan, D. and Fiat, D.- Biochim. Biophys. Acta, 800: 251 (1984).

13. Merrifield, R.B.-J. Am. Chem. Soc. 85: 2149 (1963).

14. Steinschneider, A., Burgar, M.I., Buku, A. and Fiat, D.- Int. J. Peptide Protein Res. 18: 324 (1981).

15. Ponnusamy, E., Fotadar, U., Spisni, A. and Fiat, D.- Synthesis (Submitted for publication).

16. Flouret, G.-J. Med. Chem. 13: 843 (1970).

17. Pietta, P.G., Cavallo, P.F., Takahashi, K. and Marshall, G.R.-J. Org. Chem. 39: 44 (1974).

18. Manning, J.M. -J. Am. Chem. Soc. 92: 7449 (1970).

19. Kaiser, E., Colescott, R.L., Bossinger, C.D. and Cook, P.I.-Anal. Biochem. 34: 595 (1970).