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BH_4 BIOSYNTHESIS: ^{1}H-NMR EVIDENCE FOR THE PYRUVOYL-TETRAHYDROPTERIN-SYNTHASE CATALYZED FORMATION OF 6-PYRUVOYL-TETRAHYDROPTERIN FROM DIHYDRONEOPTERIN TRIPHOSPHATE

S. Ghisla
Faculty of Biology, University of Konstanz, POB 5560, D-7750 Konstanz, FRG

S. Takikawa, P. Steinerstauch, Th. Hasler, H.-Ch. Curtius
Division of Clinical Chemistry, Department of Pediatrics, University of Zurich, Steinwiesstr. 75, CH-8032 Zurich, Switzerland

Introduction

The enzyme which catalyzes the first step in BH_4 biosynthesis from dihydroneopterin triphosphate (NH_2TP, cf (A), Fig. 1) has recently been purified to homogeneity from human liver [1,2]. The chemical structure of the product of this conversion has been deduced from its mode of formation, its chemical reactivity, and from trapping experiments [3-7] to be that of 6-pyruvoyl-tetrahydropterin (PPH_4, cf (D), Fig. 1). Up to now, however, no direct evidence for this structure has been reported. This is clearly due to the instability of PPH_4 [4,5], a molecule which might exist in several forms, derived, e.g., from cyclization of the side chain with the pterin ring. In view of the importance of this intermediate for the understanding of BH_4 biosynthesis, we have attempted to obtain direct experimental evidence for its structure by following the conversion of NH_2TP to PPH_4 with ^{1}H-NMR.

Results

NH_2TP was prepared enzymatically from GTP as described elsewhere [8]. HPLC analysis shows the presence of traces of dihydroneopterin and of some GTP, which is also seen from the ^{1}H-NMR spectrum of Fig. 1, curve A. Note that the signals of GTP are essentially unchanged after incubation (Fig. 1 (D)). Addition of PPH_4-synthase initiates marked changes in the region from 4 to 4.5 ppm. The sequence of spectra (B), (C), and (D) (Fig. 1) shows that the signals attributed to the protons at positions 7, 1', 2', and 3' of NH_2TP

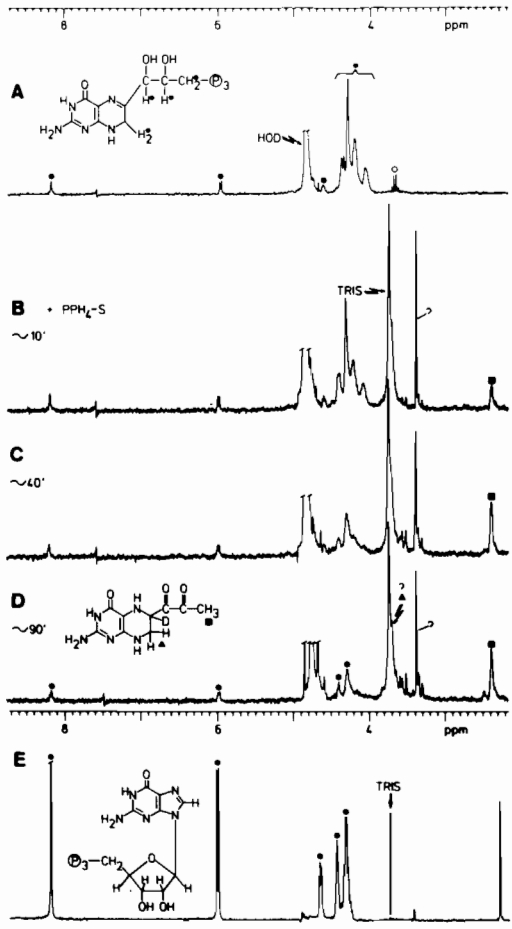

Fig. 1: Conversion of NH_2TP into PPH_4 followed by ^1H-NMR-spectroscopy. Curve (A): NH_2TP, 500 µg in 0.4 ml 7 mM phosphate buffer, pH 7.5, prepared with 99.7% D_2O (P_2O_5, Na_2CO_3). The signals marked with (•) are attributed to the protons of NH_2TP, those with (*) are from GTP (cf (E)), and (o) from a trace of ethanol (the $-CH_3$ signal at 1.22 ppm was taken as an internal standard, not shown). This solution was made anaerobic by flushing with dry N_2. ~0.3 U PPH_4S (salmon) in 0.1 ml of the same buffer (original TRIS buffer exchanged twice using an Amicon Centricon 10 membrane filter), containing 8 mM Mg^{++}, and 0.2 mM DTE, were then added, and recording of spectrum (B) started immediately (~100 scans, total acquisition time ~15 min, times shown are the average of start/end of acquisition). The signal at 3.7 ppm corresponds to TRIS (contaminant, original PPH_4S buffer), and that at 3.35 ppm is probably a trace of methanol. Neither signal was present in duplicate intensity during the reaction. Spectrum (D) was recorded after warming the sample at 37°C for 20 min and re-recorded with a Bruker 250 MHz instrument at 25°C. The symbols refer to the protons shown on the structures.

gradually disappear. The remaining minor signals at 4.2 - 4.5 ppm seen on spectrum (D) are due in part to traces of dihydroneopterin. Concomitantly a new signal appears at ~2.4 ppm; its intensity is maximal at (C) and then decreases. The chemical shift of this peak is compatible with the presence of a $-C(=O)-CH_3$ group. The integration of this signal clearly is too small to account for three 3'-protons in PPH_4. The answer to this apparent inconsistency is given by the GC-MS spectra of Fig. 2, which were obtained from the

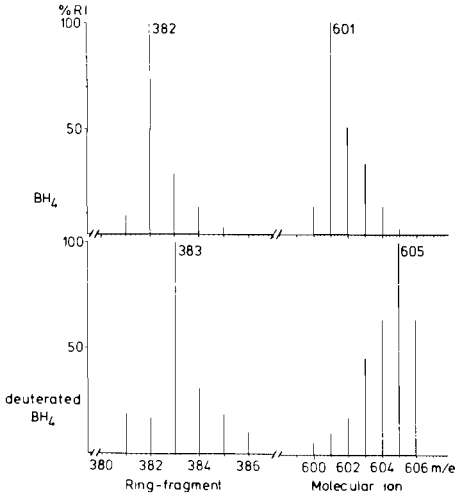

Fig. 2: Mass spectra of authentic BH_4 and BH_4 obtained from the reduction of PPH_4 originating from the experiment of Fig. 1. Fig. 2 shows the introduction of one deuterium into the ring fragment and three into the side chain. An aliquot (200 µl) of the sample used for the NMR measurements was incubated for 30 min at 37°C with ~30 mU sepiapterin reductase, 15 mM NADPH, 12.5 mM phosphate buffer, pH. 6.5, in a total volume of 400 µl. The trimethylsilylated BH_4 sample was analyzed for deuterium content with GC-MS, as described by T. Kuster and A. Niederwieser (9).

sample of spectrum (D) (Fig. 1), and which clearly show that one deuterium has been introduced into the pterin ring, most probably at position 6, and that position 3' is deuterated to 70%. This exchange has been observed earlier [3], and is likely to be catalyzed by PPH_4-synthase since pyruvate does not exchange under similar conditions. At ~3.7 ppm, masked in part by the peak attributed to TRIS, a signal is apparent as a shoulder, which can be attributed tentatively to the $7-CH_2$ function (C(7)-H of model tetrahydropterins have signal(s) at 3.2 - 3.7 ppm). The $C(7)-H_2$ signal of PPH_4 should appear as an AB system. (α to asymmetric C(6)). At longer times of incubation several minor unidentified signals also appear (e.g., at ~2.5 ppm, and from 3.3 to 3.8 ppm), which are attributed to the expected decay products of PPH_4.

Conclusions

The NMR spectra provide the first direct evidence that PPH_4-synthase catalyzes chemical transformations involving all hydrogens of the biopterin side chain. The chemical shift of the new signal at ~2.4 ppm is compatible with the presence of a carbonyl group at its α-position. Similarly, the disappearance of the C(7)-H_2 signal (4.2 - 4.4 ppm), i.e., its shift to ~3.7 ppm is compatible with the reduction of the neighboring imine (>C=N- ⟶ >CH-NH-), that is, with the conversion of a dihydro- to a tetrahydropterin species. The rapid exchange of the protons of 3'-CH_3 suggests that the same base which is presumably catalyzing the abstraction of the 1', and 2' hydrogens, is also capable of attacking the 3'-CH_3, thus promoting enolization and subsequent exchange. The present results are in agreement with the biosynthetic mechanism proposed earlier by our [1-3] and by other groups [4-7].

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References

1. Takikawa, S., H.-Ch. Curtius, U. Redweik, and S. Ghisla. 1986. Biochem. Biophys. Res. Commun. 134, 646-651

2. Takikawa, S., H.-Ch. Curtius, U. Redweik, W. Leimbacher, and S. Ghisla. Eur. J. Biochem., (submitted)

3. Curtius, H.-Ch., D. Heintel, S. Ghisla, T. Kuster, W. Leimbacher, and A. Niederwieser. 1985. Eur. J. Biochem. 148, 413-419

4. Masada, M., M. Akino, T. Sueoka, and S. Katoh. 1985. Biochem. Biophys. Acta 840, 235-244

5. Switchenko, A.C. and G.M. Brown. 1985. J. Biol. Chem. 260, 2945-2451

6. Smith, G.K. and C.A. Nichol. 1986. J. Biol. Chem. 261, 2725-2737

7. Milstien, S. and S. Kaufman. 1985. Biochem. Biophys. Res. Commun. 128, 1099-1107

8. Heintel, D., S. Ghisla, H.-Ch. Curtius, A. Niederwieser, and R.A. Levine. 1984. Neurochem. Int. 6, 141-155

9. Kuster, T. and A. Niederwieser. 1983. J. Chromatogr. 278, 245-254