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Random decarboxylation of uroporphyrinogen III by human hepatic uroporphyrinogen decarboxylase

JINLI LUO and C. K. LIM*

Division of Clinical Cell Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)

ABSTRACT

The type III heptacarboxylic porphyrinogens derived from enzymic decarboxylation of an acetic acid substituent on uroporphyrinogen III to a methyl group by human hepatic uroporphyrinogen decarboxylase has been analysed by reversed-phase high-performance liquid chromatography with electrochemical detection. The results showed that all four possible heptacarboxylic acid porphyrinogen isomers, with the methyl group attached to rings A, B, C and D of the tetrapyrrole macrocycle, respectively, were formed in almost equal proportions. It was concluded that the normal pathway of uroporphyrinogen III decarboxylation in human liver follows a random mechanism.

INTRODUCTION

Uroporphyrinogen decarboxylase (EC 4.1.1.37) catalyses the conversion of uroporphyrinogen III into coproporphyrinogen III. The reaction is stepwise with heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrinogens as intermediates [1–3]. Nuclear magnetic resonance spectroscopic analyses [2] showed that the predominant intermediates excreted in the urine and faeces of rats poisoned with hexachlorobenzene were the heptacarboxylic porphyrin with the methyl group located at ring D (7d)[a], hexacarboxylic prophyrin with the two methyl groups attached on rings A and D (6ad)[a], and pentacarboxylic porphyrin with the three methyl groups situated at rings A, B and D (5abd)[a]. It was therefore reasonable to assume that the preferred route to coproporphyrinogen III is by an orderly, clockwise decarboxylation sequence starting from the ring D acetic acid group of uroporphyrinogen III and proceeding through the acetic acid groups on rings A, B and C, respectively [2]. Analyses of porphyrin intermediates in normal human urine by high-performance liquid chromatography (HPLC), however, showed the presence of all isomeric forms and therefore indicated a random decarboxylation mechanism [4,5]. The situation is further complicated by the fact that in porphyria cutanea tarda (PCT), the heptacarboxylic porphyrin

[a] The letters a, b, c and d denote the position of the methyl group on rings A, B, C and D, respectively, *i.e.* the position in which the acetic acid group has been decarboxylated [2].

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found in the urine and faeces was mainly 7d while all four type III pentacarboxylic porphyrins (5abc, 5abd, 5acd and 5bcd) were detected in virtually equal proportions. Since it has already been shown [6] that enzymic decarboxylation of uroporphyrinogen III by erythrocyte uroporphyrinogen decarboxylase produced all four type III heptacarboxylic porphyrinogen isomers (7a, 7b, 7c and 7d) (Fig. 1), it is possible that the 7d present in the urine and faeces of PCT was derived from the liver, which is the most affected site in this disease, by a different decarboxylation mechanism. This paper describes the detailed HPLC analysis of the heptacarboxylic porphyrinogen isomers formed by decarboxylation of uroporphyrinogen III in normal human liver biopsy homogenates and provides further evidence to support a random decarboxylation mechanism.

EXPERIMENTAL

Materials and reagents

Uroporphyrin III was from Porphyrin Products (Logan, UT, U.S.A.). A standard mixture of heptacarboxylic prophyrins containing 7a, 7b, 7c and 7d was prepared and isolated as previously described [7]. Ammonium acetate, glacial acetic acid, dimethyl sulphoxide (DMSO), concentrated hydrochloric acid, concentrated sulphuric acid, disodium ethylenediaminetetraacetic acid (EDTA), mercury, sodium, potassium hydroxide, dipotassium hydrogenphosphate, trichloroacetic acid and Triton X-100 were AnalaR grade from BDH (Poole, U.K.). Tris and dithiothreitol (DTT) were from Sigma (Poole, U.K.). Acetonitrile and methanol were HPLC grade from Rathburn (Walkerburn, U.K).

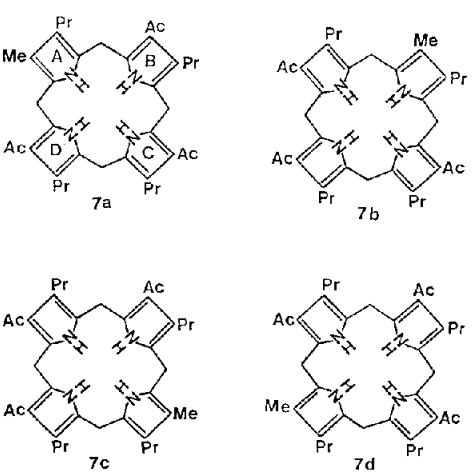

Fig. 1. Structures of type III heptacarboxylic porphyrinogen isomers. Ac represents an acetic acid group and Pr represents a propionic acid group.

Preparation of porphyrinogens

Uroporphyrin III or a mixture of heptacarboxylic porphyrins was dissolved in 0.01 *M* potassium hydroxide flushed with nitrogen and vortex-mixed with 3% (w/w) sodium amalgam until no fluorescence was observed under UV light. The porphyrinogen solution was transferred quickly into a clean tube and was used immediately.

Preparation of human liver homogenates

Normal human tissues were obtained from patients investigated for hepatomegaly, pyrexia of unknown origin or hyperbilirubinaemia (Gilbert's syndrome) and were found to be normal. They were obtained with a Menghini needle via the subcostal route under local anaesthetic. The tissue (200 mg) was homogenised in 2 ml of 0.1 *M* potassium phosphate buffer containing 0.1 m*M* disodium EDTA, 0.1% Triton X-100 and 10 m*M* DTT, pH 6.8, in a 5-ml glass homogenizer (Jencons Scientific, Leighton Buzzard, U.K.).

Enzyme incubation procedure

Human liver homogenate (2 ml) was preincubated at 37°C for 5 min in a water bath in the dark. Uroporphyrinogen III (300 μl, 4 μM) was then added. The tube was flushed with nitrogen, mixed thoroughly, stoppered and incubated for 30 min at 37°C. The reaction was terminated by vortex-mixing with 10 ml of 10% trichloroacetic acid–DMSO (1:1, v/v). The mixture was exposed to light from a 60-W bulb for 1 h to oxidise the porphyrinogens into porphyrins and then centrifuged at 2500 *g* for 10 min. The supernatant was transferred into a clean tube.

Isolation of type III heptacarboxylic porphyrins by HPLC

The supernatant from the above enzyme reaction mixture was diluted with 5 volumes of 1 *M* ammonium acetate buffer, pH 5.16. The solution was then loaded into a C_{18} Bond Elut sorbent extraction cartridge (Jones Chromatography, Hengoed, U.K.) which had been preconditioned by washing successively with 2 ml of methanol and 10 ml of 1 *M* ammonium acetate buffer, pH 5.16. The cartridge was washed with 10 ml of 1 *M* ammonium acetate buffer, pH 5.16, to remove some early eluting impurities, and the adsorbed prophyrins were recovered by elution with four 5-ml volumes of 10% (v/v) acetonitrile in methanol. The eluates were pooled and concentrated to about 0.5 ml at 35°C under nitrogen for HPLC separation.

The liquid chromatograph consisted of a Varian (Walton-on-Thames, U.K.) Model 5000 solvent delivery system, a Rheodyne (Cotati, CA, U.S.A.) 7125 injector fitted with a 200-μl loop and a Varian UV-100 variable-wavelength detector set at 400 nm for detection. The separation was performed on a 250 mm × 5.0 mm I.D. Hypersil ODS (5 μm particle size) column (Shandon Scientific, Runcorn, U.K.) by gradient elution with the mobile phase system previously described for the separation of porphyrin isomers, including heptacarboxylic porphyrin I and

III isomers [8]. The peak corresponding to type III heptacarboxylic porphyrin was collected and pooled. The organic solvent in the eluate was removed by evaporation under nitrogen and the porphyrin in the aqueous solution was extracted on a C_{18} Bond Elut cartridge and recovered by elution with 10% acetonitrile in methanol as described above.

Analysis of heptacarboxylic porphyrinogen isomers by HPLC

The heptacarboxylic porphyrin III isolated from the enzyme reaction mixture was reduced to the corresponding porphyrinogen for isomer composition analysis by HPLC. The separation was carried out on a 150 mm × 4.6 mm I.D. Asahipak ODP-50 (5 μm particle size) column (Asahi, Kawasaki-shi, Japan) with acetonitrile–methanol–1 *M* ammonium acetate, pH 5.16 (7:3:90, v/v) containing 0.27 m*M* EDTA as mobile phase. The flow-rate was 0.5 ml/min. The mobile phase was degassed with a stream of helium before use, and degassing was continued during the separation. An electrochemical detector (LCA-15 from EDT Research, London, U.K.) set at an operation potential of + 0.65 V was used for solute detection.

RESULTS AND DISCUSSION

Although it has been shown that the first step of enzymic decarboxylation of uroporphyrinogen III in the erythrocytes of normal subjects and of patients with sporadic PCT is random [6], it does not explain why the heptacarboxylic porphyrin 7d accumulates in the urine and faeces of PCT patients. It is well known that in sporadic PCT, uroporphyrinogen decarboxylase defect is confined to the liver with normal enzyme activity being found in the erythrocytes [9,10]. It is therefore possible that the mechanism of decarboxylation in red cells is different from that in the liver, with the latter following a clockwise sequence starting at the ring D acetic acid group of uroporphyrinogen III [2] leading to the accumulation and excretion of 7d. In other words, 7d was of hepatic origin. Analysis of the heptacarboxylic porphyrinogen isomers produced by decarboxylation of uroporphyrinogen III in the liver homogenate should allow this possibility to be tested.

In order to eliminate the possibility that isomerization of porphyrinogen had taken place during the incubination and particularly during the oxidation and extraction stages, the heptacarboxylic porphyrinogen 7d was incubated without liver tissue and then similarly oxidised and extracted. Analysis by HPLC showed no isomerization and 7d was recovered unchanged.

The separation of a standard mixture and of the heptacarboxylic porphyrinogen isomers derived from decarboxylation of uroporphyrinogen III by hepatic uroporphyrinogen decarboxylase is shown in Fig. 2a and b, respectively. The result unequivocally demonstrated that, similar to erythrocyte uroporphyrinogen decarboxylase, the first step of decarboxylation of uroporphyrinogen III in normal subjects is random in nature. Unfortunately liver tissues from patients with

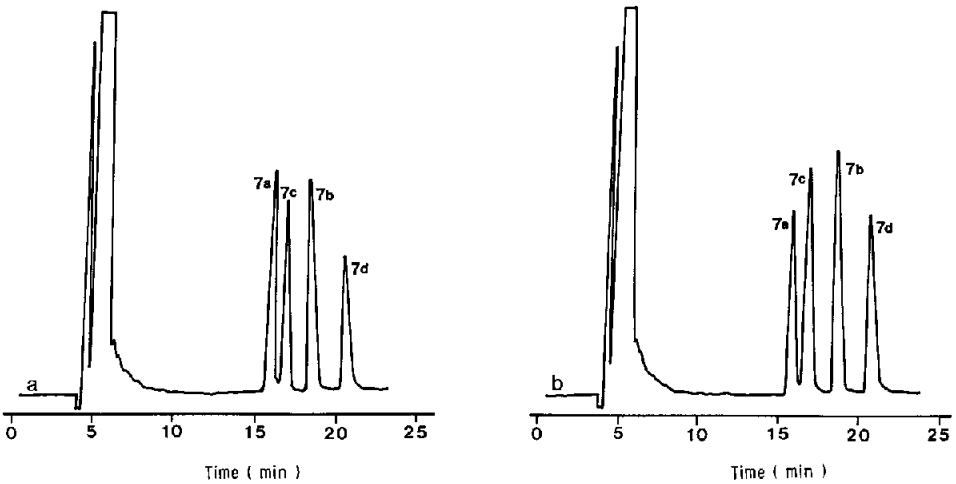

Fig. 2. HPLC separation of heptacarboxylic porphyrinogen isomers. (a) Standard mixture; (b) enzyme incubation mixture of normal subjects. HPLC conditions are given in the Experimental section.

PCT were not available for the present study. It therefore remains uncertain whether the accumulation of 7d in PCT is due to a preferential decarboxylation of the ring D acetic acid group of uroporphyrinogen III or because the defective enzyme in the liver is unable to metabolise 7d effectively. To answer these questions further experiments with liver tissues from PCT patients are required. It is also necessary to study the subsequent steps of decarboxylation with individual porphyrinogen isomers as substrates.

REFERENCES

1 J. M. Tomio, R. G. Garcia, L. C. San Martin de Viale and M. Grinstein, *Biochim. Biophys. Acta*, 198 (1970) 353.
2 A. H. Jackson, H. A. Sancovich, A. M. Ferramola, N. Evans, D. E. Games, S. A. Matlin, G. H. Elder and S. G. Smith, *Philos. Trans. R. Soc. London Ser. B.*, 273 (1976) 191.
3 J. P. Kushner, A. J. Barbuto and G. R. Lee, *J. Clin. Invest.*, 58 (1976) 1089.
4 C. K. Lim and J. M. Rideout, *J. Liq. Chromatogr.*, 6 (1983) 1969.
5 C. K. Lim, J. M. Rideout and D. J. Wright, *J. Chromatogr.*, 282 (1983) 629.
6 J. Luo and C. K. Lim, *Biochem. J.*, 268 (1990) 515.
7 C. K. Lim, F. Li and T. J. Peters, *Biochem. J.*, 247 (1987) 229.
8 C. K. Lim, J. M. Rideout and D. J. Wright, *Biochem. J.*, 211 (1983) 435.
9 G. H. Elder, G. B. Lee and J. A. Tovey, *N. Engl. J. Med.*, 229 (1978) 274.
10 H. de Verneuil, G. Aitken and Y. Nordmann, *Hum. Genet.*, 44 (1978) 145.