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Decarboxylation of uroporphyrinogen III by erythrocyte uroporphyrinogen decarboxylase

Evidence for a random decarboxylation mechanism

Jinli LUO and Chang Kee LIM*
Division of Clinical Cell Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, U.K.

The isomeric composition of type-III heptacarboxylic porphyrinogens derived from decarboxylation of uroporphyrinogen III by erythrocyte uroporphyringogen decarboxylase was analysed by h.p.l.c. with electrochemical detection. All four possible isomers were identified, and there were little differences in the proportion of isomers formed by erythrocytes from normal subjects and from patients with sporadic porphyria cutanea tarda. The results provide conclusive evidence that the normal decarboxylation pathway is random in nature, and the fourth isomer only increases when enzyme abnormality is found.

INTRODUCTION

Uroporphyrinogen decarboxylase (EC 4.1.1.37) is a cytosolic enzyme of the haem-biosynthetic pathway that catalyses the decarboxylation of four side-chain acetate groups of uroporphyrinogen III to coproporphyrinogen III (Manzerall & Granick, 1958). The reaction is stepwise, with hepta-, hexa- and penta-carboxylic porphyrinogens as the intermediates (Tomio *et al.*, 1970; Jackson *et al.*, 1976; Kushner *et al.*, 1976). There are four possible heptacarboxylic porphyrinogen isomers (Fig. 1) that may be formed by the enzyme reaction, and evidence from the urine and faeces of hexachlorobenzene-poisoned rats and from the urine of patients with porphyria cutanea tarda (PCT) have shown that about 98% of the compound had the **7d** structure (Jackson *et al.*, 1976). This led to the suggestion that the preferred pathway of decarboxylation is clockwise, starting from the ring-D acetate group of uroporphyrinogen III and then proceeded around the macrocycle in an orderly fashion through the acetate groups of rings A, B and C (Jackson *et al.*, 1976). The detailed analysis of the hexa- and penta-carboxylic porphyrins in normal and PCT urine by h.p.l.c., however, showed the presence of all possible isomeric forms (Lim & Rideout, 1983; Lim *et al.*, 1983*a*) in proportions inconsistent with an ordered sequential mechanism.

The present paper describes the isolation and identification of heptacarboxylic porphyrinogen isomers derived from decarboxylation of uroporphyrinogen III by erythrocyte uroporphyrinogen decarboxylase and provides conclusive evidence for a random, rather than an ordered, decarboxylation sequence.

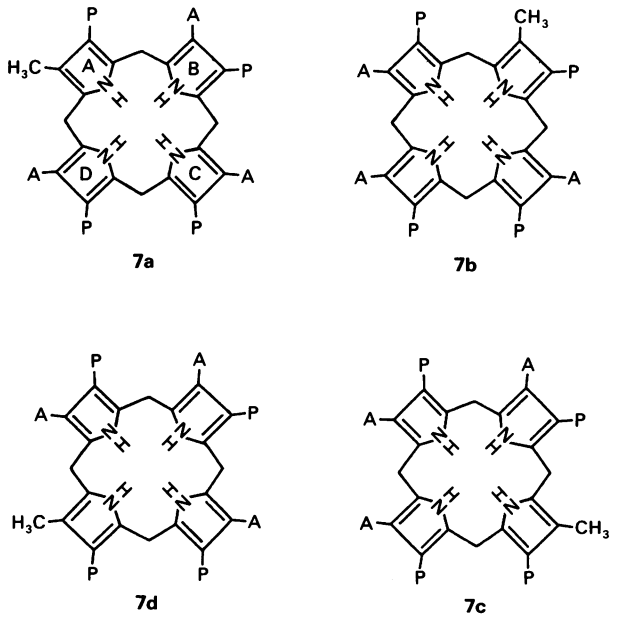

Fig. 1. Structures of type-III heptacarboxylic porphyrinogen isomers

The letters **a**, **b**, **c** and **d** denote the position of the methyl group, i.e. the position in which the acetic acid group has been decarboxylated (Jackson *et al.* 1976). 'A' represents an acetic acid group, and 'P' represents a propionic acid group.

EXPERIMENTAL

Materials and reagents

Uroporphyrin III was from Porphyrin Products (Logan, UT, U.S.A.) and was purified by h.p.l.c. before use. A standard mixture of type-III heptacarboxylic porphyrin (containing isomers **7a**, **7b**, **7c** and **7d**; see Fig. 1) was prepared by heating uroporphyrin III in 0.5 M-HCl, and methyl esters were isolated by t.l.c. as previously described (Lim *et al.*, 1987). The esters were hydrolysed in 25% (w/v) HCl at room temperature in the dark for 96 h and then extracted at pH 3.5 into ethyl acetate (Lim *et al.*, 1987).

Ammonium acetate, acetic acid, concentrated HCl, concentrated H_2SO_4, dimethyl sulphoxide (DMSO), K_2HPO_4, EDTA disodium salt, ethyl acetate, metallic sodium, mercury, KOH, trichloroacetic acid and Triton X-100 were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. Dithiothreitol (DTT) and Tris were from Sigma Chemical Co., Poole, Dorset, U.K. Acetonitrile and methanol were h.p.l.c. grade from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.

Bond Elut disposable sorbent extraction cartridges (C_{18}) were

Abbreviations used: DMSO, dimethyl sulphoxide; DTT, dithiothreitol; PCT, porphyria cutanea tarda.

* To whom correspondence should be addressed.

from Jones Chromatography Ltd., Hengoed, Mid-Glamorgan, Wales, U.K.

Preparation of red blood cells

Blood was collected in heparinized tubes and centrifuged at 2500 *g* for 15 min at 4 °C. The plasma and leucocytes were discarded. The red cells were washed three times with cold iso-osmotic saline (0.9% NaCl) and were centrifuged after each washing. The packed red cells were used immediately for the enzyme reaction.

Preparation of porphyrinogens

Uro- or heptacarboxylic porphyrins were dissolved in 0.01 M-KOH in an N_2-filled tube and shaken vigorously with 3% (w/v) sodium amalgam until no fluorescence was detected under a u.v. lamp. The porphyrinogen solution was transferred into a clean tube, flushed with N_2 and kept on ice in the dark if not used immediately. It was stable for at least 2 h.

Incubation medium for the uroporphyrinogen decarboxylase reaction

This was either 0.5 M Tris/HCl buffer, pH 6.8 containing 150 μM-EDTA and 0.1% (w/v) Triton X-100 or 0.1 M-K_2HPO_4, pH 6.8, containing 0.1 mM-EDTA and 0.1% Triton X-100. DTT, at 156 and 10 mM, was added to the Tris/HCl and phosphate buffer respectively, just before the enzyme reaction was initiated.

Enzyme incubation procedure

Red cells (400 μl) were thoroughly mixed with 8 ml of incubation medium and preincubated at 37 °C for 5 min in a water bath in the dark. Uroporphyrinogen III (800 μl, 3.7 μM) was then added and, after mixing, the tube was flushed with N_2, stoppered and incubated for 30 min at 37 °C. The reaction was terminated by vortex-mixing with 40 ml of 10% (w/v) trichloroacetic acid/DMSO (1:1, v/v). The mixture was exposed to light from a 60 W bulb for 1 h to oxidize the porphyrinogens and then centrifuged at 2500 *g* for 10 min. The supernatant was transferred into a clean tube. To determine whether isomerization can take place, the heptacarboxylic porphyrinogen (**7d**) was incubated with boiled red cells in parallel and then subjected to the same oxidation and extraction processes.

Isolation of type-III heptacarboxylic porphyrin from the enzyme incubation mixture

The supernatant from the above enzyme reaction mixture was diluted with 5 vol. of 1 M-ammonium acetate buffer, pH 5.16, and then loaded into a C_{18} Bond Elut sorbent extraction cartridge which had been preconditioned successively with methanol (2 ml) and 10 ml of ammonium acetate buffer (1 M, pH 5.16). After the cartridge had been washed with 10 ml of the ammonium acetate buffer, the adsorbed porphyrins were recovered by elution with 4 × 5 ml of 10% (v/v) acetonitrile in methanol. The eluate was concentrated to about 0.5 ml at 30 °C under N_2 and the porphyrins in this solution were separated by h.p.l.c. with the system previously described for the separation of porphyrin isomers (Lim *et al.*, 1983*b*). The peak corresponding to type-III heptacarboxylic porphyrin was collected and pooled. The acetonitrile in the eluate was removed by evaporation under N_2, and the porphyrin in the aqueous solution was concentrated and recovered by the sorbent extraction technique described above.

H.p.l.c. of heptacarboxylic porphyrinogens

A Varian (Walton-on-Thames, Surrey, U.K.) model-5000 liquid chromatograph was used. Sample injection was by a Rheodyne (Cotati, CA, U.S.A.) 7125 injector fitted with a 100 μl loop. The porphyrinogens were separated on a Asahipak ODP-50 column (15 cm × 4.6 mm internal diameter; 5 μm particle size) from Asahi Chemical Industry Co., Kawasaki-shi, Japan. The mobile phase was acetonitrile/methanol/1 M-ammonium acetate buffer, pH 5.16 (7:3:90, by vol.) containing 0.27 mM-EDTA. The flow rate was 0.5 ml/min. The mobile phase was thoroughly degassed with a stream of helium before use, and degassing was continued during the separation. The porphyrinogens were detected with a LCA-15 electrochemical detector (EDTA Research, London N.W.10, U.K.) set at an operation potential of +0.65 V. The detector sensitivity was either 10 or 30 nA.

RESULTS AND DISCUSSION

Although analyses of the hexa- and penta-carboxylic porphyrins in urine have indicated a random decarboxylation mechanism (Lim & Rideout, 1983; Lim *et al.*, 1983*a*). these intermediates with lower number of acetate groups could have

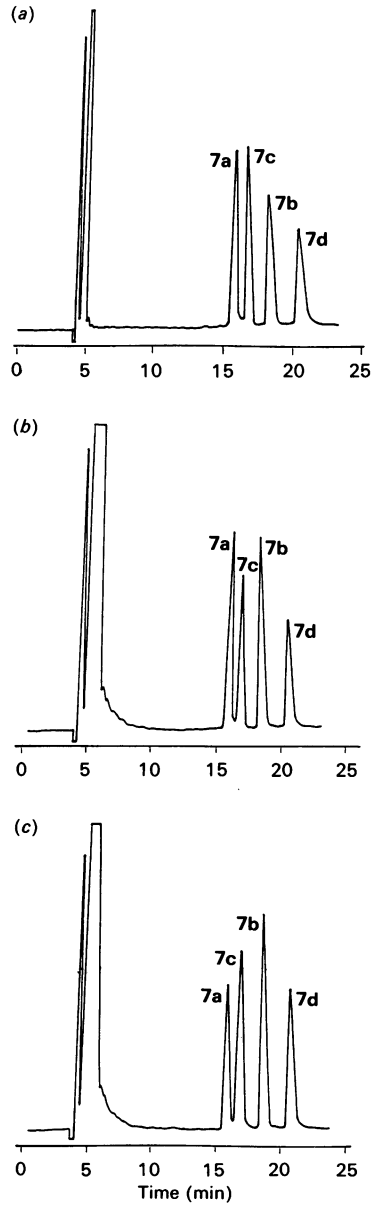

Fig. 2. H.p.l.c. separation of heptacarboxylic porphyrinogen isomers

(*a*) A standard mixture; (*b*) and (*c*) from the enzyme incubation mixtures of normal subjects and sporadic-PCT patients respectively.

Table 1. Erythrocyte uroporphyrinogen decarboxylase activities and proportions of 7a, 7b, 7c and 7d formed in control subjects and PCT patients

The substrate concentration was 3.7 mM, and incubation was for 30 min at 37 °C. Results are means ± S.D.

	n	Enzyme activity (nmol of heptacarboxylic porphyrin III/h per ml of red cells)	Isomer...	Proportion (%) 7a	7b	7c	7d
Control subject	12	8.9±1.2		27±2	29±3	23±4	21±2
Sporadic PCT	4	8.6±0.8		24±2	28±2	26±3	22±3

been minor products which have accumulated because of differences in enzyme kinetics. A better solution to the conflicting evidence on the nature of uroporphyrinogen III decarboxylation is to analyse and identify positively the isomeric form(s) of the first decarboxylation intermediate, i.e. heptacarboxylic porphyrinogen(s) produced by the uroporphyrinogen decarboxylase reaction. Previous studies have shown that, at high substrate concentrations, uroporphyrinogen III was converted mainly into heptacarboxylic porphyrinogen by uroporphyrinogen decarboxylase (Straka & Kushner, 1983; Kawanishi *et al.*, 1983). By using the sorbent extraction technique, it is therefore possible to isolate enough heptacarboxylic porphyrin from the enzyme reaction mixture for isomer-composition analysis. There was no difference in product formation whether phosphate buffer, pH 6.8, or Tris/HCl buffer, pH 6.8, was used as the incubation medium.

The heptacarboxylic porphyrinogens were separated on a polymeric reversed-phase gel modified with octadecyl carbon group (C_{18}) on a hard polymer skeleton (Asahipak ODP-50) with the mobile phase system described previously for the separation of heptacarboxylic porphyrinogens on ODS-Hypersil (Lim *et al.*, 1987). Similar resolution was obtained, but the polymer-based column has the advantage of being stable in the pH range 2–13. This allows the repeated analysis of porphyrinogens in alkaline (0.01 M-KOH) solution without the column being damaged by the high pH as would have been the case if a silica-based material were used. The separation of a standard mixture, and of the heptacarboxylic porphyrinogens formed by the red cells of a normal subject and a patient with sporadic PCT, are shown in Figs. 2(*a*), 2(*b*) and 2(*c*) respectively. In all cases four possible isomers (**7a**, **7b**, **7c** and **7d**) were detected. There was little difference between the proportions of isomers formed in normal subjects and those formed in sporadic-PCT patients with normal red-cell uroporphyrinogen decarboxylase activity (Table 1). Individual variation in isomeric composition was observed, but **7d** was never the major isomer formed. Heptacarboxylic porphyrinogen III production was linear for up to 60 min, and it increased with increasing substrate concentration from 0.5 to 6 μM. There were insignificant changes in isomer composition with incubation time and substrate concentrations. There were also insignificant differences in the rates of conversion of **7a**, **7b**, **7c** and **7d** into porphyrinogens with lower number of acetate groups when these were incubated with normal red cells. These results showed conclusively that decarboxylation of uroporphyrinogen III by red-cell uroporphyrinogen decarboxylase is random in nature. To confirm that the isomers were not derived from one or more isomers by acid-catalysed isomerization, pure **7d** was incubated with boiled red cells and then similarly oxidized and extracted for h.p.l.c. analysis. No isomerization was observed, and the compound was recovered unchanged.

The heptacarboxylic porphyrin isolated from the faeces of rats poisoned with hexachlorobenzene and from the urine of patients with PCT was predominantly **7d** (Jackson *et al.*, 1976). This could also be confirmed by h.p.l.c. analysis of the corresponding heptacarboxylic porphyrinogen (Lim *et al.*, 1987). Since **7d** appears to accumulate in tissues where uroporphyrinogen decarboxylase activity is low, it is probable that the **7d** found in urine of PCT patients was of hepatic origin, since the liver is the most affected site. Whether this is a consequence of preferred decarboxylation of the ring-D acetic acid group of uroporphyrinogen III in PCT and hexachlorobenzene-poisoned rat or because enzyme abnormality in the liver led to selective inhibition and therefore accumulation of **7d** is not known.

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