Order of uroporphyrinogen III decarboxylation on incubation of porphobilinogen and uroporphyrinogen III with erythrocyte uroporphyrinogen decarboxylase

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The isomeric compositions of the heptacarboxylic, hexacarboxylic and pentacarboxylic porphyrinogens formed by incubation of porphobilinogen with human red-cell haemolysates have been analysed and compared with those derived from incubation with chemically prepared uroporphyrinogen III as substrate. The results indicated that when supplied with an excess (3.7 μM) of exogenous uroporphyrinogen III, uroporphyrinogen decarboxylase utilized the substrate at random and a mixture of isomers was produced; whereas with uroporphyrinogen III generated enzymically from porphobilinogen as substrate a clockwise decarboxylation sequence was observed, resulting in the formation of intermediates mainly with the ring-D, rings-AD and rings-ABD acetate groups decarboxylated. Using [^{14}C]uroporphyrinogen III as substrate at low concentrations (0.01–0.5 μM) also led to preferential decarboxylation of the ring-D acetate group. It was concluded that the order of uroporphyrinogen III decarboxylation is substrate-concentration-dependent, and under normal physiological conditions enzymic decarboxylation is most probably orderly and clockwise, starting at the ring-D acetate group.

INTRODUCTION

Uroporphyrinogen decarboxylase (EC 4.1.1.37) is a cytosolic haem-biosynthetic enzyme that catalyses the stepwise decarboxylation of the acetate groups on the rings A, B, C and D of uroporphyrinogen III to coproporphyrinogen III with hepta-, hexa- and penta-carboxylic porphyrinogens as intermediates (Mauzerall and Granick, 1958; Tomio et al., 1970; Jackson et al., 1976). One of the outstanding questions concerning this enzyme that remains to be answered is whether the reaction proceeds via a preferred route that begins at ring D, through rings A and B, and ends at ring C (Figure 1) or is able to begin and end at any ring acetate group. On the one hand, the intermediates isolated from the faeces of hexachlorobenzene-poisoned rats, which are uroporphyrinogen decarboxylase-deficient, were 7d, 6ad and 5abd (Figure 1) respectively, consistent with a clockwise route starting at ring D (Jackson et al., 1976). On the other hand, mixtures of isomeric intermediates have been detected in human urine (Jackson et al., 1980; Lim and Rideout, 1983) and the heptacarboxylic porphyrinogen formed by incubation of uroporphyrinogen III with red-cell haemolysates was a mixture of the four possible isomers (Lash, 1979; Luo and Lim, 1990). This indicated a random decarboxylation route.

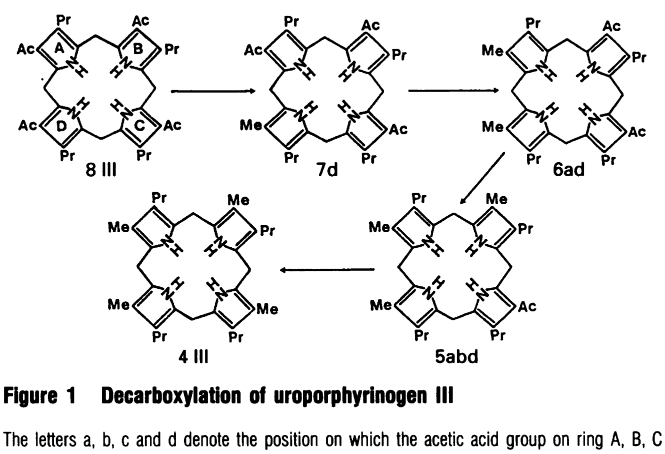

Figure 1 Decarboxylation of uroporphyrinogen III

The letters a, b, c and d denote the position on which the acetic acid group on ring A, B, C and D respectively has been decarboxylated to a methyl group.

It has been suggested recently (Lash, 1991) that two different routes of decarboxylation may be operating depending on whether the substrate is the intact uroporphyrinogen III–uroporphyrinogen III synthase complex which is presented to uroporphyrinogen decarboxylase in a specific orientation or free uroporphyrinogen III leaked from such an enzyme complex which is then presented to the decarboxylase at random. To test the hypothesis the present paper describes the detailed analysis of the hepta-, hexa- and penta-carboxylic porphyrinogens formed by incubation of porphobilinogen (PBG) with human red-cell haemolysates and compared with results previously obtained with uroporphyrinogen III as substrate (Lash, 1979; Luo & Lim, 1990).

EXPERIMENTAL

Materials and reagents

Porphobilinogen (PBG), uroporphyrin III, dithiothreitol (DTT) and Triton X-100 were from Sigma Chemical Co. (Poole, Dorset, U.K.). A standard mixture of type III heptacarboxylic porphyrin containing isomers 7a, 7b, 7c and 7d was prepared by heating uroporphyrin III in 0.5 M HCl and isolated as previously described (Lim et al., 1983). 5-Amino[4-^{14}C]laevulinate (sp. radioactivity 50 Ci/mol) used for the enzymic generation of [^{14}C]-uroporphyrin III (Smith & Francis, 1981) was from The

Abbreviations used: DTT, dithiothreitol; PBG, porphobilinogen.
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Radiochemical Centre, Amersham, Bucks., U.K. The [^{14}C]uroporphyrin III was purified by h.p.l.c. before use. Ammonium acetate, acetic acid, trichloroacetic acid, conc. HCl, I_2, $MgCl_2$, K_2HPO_4, KH_2PO_4 and Tris/HCl were AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile and methanol were h.p.l.c. grade from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.).

Incubation of [^{14}C]uroporphyrinogen III with human red-cell haemolysates

Washed red cells (100 μl) were thoroughly mixed with 2 ml of 0.05 M Tris/HCl buffer, pH 6.8, containing 1.5 g/l $MgCl_2$, and 1 ml/l Triton X-100 or 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 6.8, containing 150 μM EDTA and 1 ml/l Triton X-100. DTT (10 mM) was added to buffer before the enzyme reaction was initiated. Uroporphyrinogen III (200 μl; 0.01–0.05 μM) was then added and mixed well. The mixture was flushed with N_2 and incubated for 60 min at 37 °C in the dark and then analysed by h.p.l.c., the procedures for which are given in detail under 'H.p.l.c.', 'H.p.l.c. of heptacarboxylic porphyrinogens' and 'Radioactivity counting' below.

Incubation of PBG with human red-cell haemolysates

Washed red cells (30 μl) were thoroughly mixed with 1 ml of 0.05 M Tris/HCl buffer, pH 7.25 containing 1.5 g/l $MgCl_2$ and 1 ml/l Triton X-100. The mixture was flushed with N_2 and pre-incubated for 5 min at 37 °C in the dark, and then 5–50 μl (10–100 μg) of PBG substrate was added. The incubation was carried out for 60 min at 37 °C in the dark. The reaction was terminated by vortex-mixing the assay mixture with 1.5 ml of cold 10% trichloroacetic acid containing 0.5% (w/v) I_2. The mixture was centrifuged at 2000 g for 10 min at 4 °C and the supernatant containing the porphyrins was separated by h.p.l.c.

H.p.l.c.

A Varian Associates (Walnut Creek, CA, U.S.A.) model-5000 liquid chromatograph was used with a Perkins–Elmer (Beaconsfield, Bucks., U.K.) LS-3 fluorescence detector set at excitation and emission wavelengths of 405 and 618 nm respectively. The sample (500 μl portions of the supernatant from the incubation mixture) was injected with a Rheodyne (Cotati, CA, U.S.A.) injector fitted with a 500 μl loop. The separation was carried out on a 250 mm × 5 mm (int.diam.) Hypersil-ODS column (Shandon Scientific, Runcorn, Cheshire, U.K.) with the gradient elution system described for the separation of porphyrin isomers (Lim et al., 1983) as follows. Solvent A consisted of 10% acetonitrile in 1 M ammonium acetate buffer, pH 5.16; solvent B consisted of 10% acetonitrile in methanol. Gradient elution was from 0 to 70% in 30 min. The peaks corresponding to hepta-, hexa- and penta-carboxylic porphyrins respectively were collected and concentrated by solid-phase extraction (Luo and Lim, 1990) for isomer composition analysis.

H.p.l.c. of heptacarboxylic porphyrinogens

The heptacarboxylic porphyrins were reduced to porphyrinogens in 0.01 M KOH by shaking vigorously with 3% (w/w) sodium amalgam until no porphyrin fluorescent was detectable under a u.v. lamp. The porphyrinogens were then separated on a Asahipak ODP-50 column [150 mm × 4.6 mm (int.diam.); 5 μm particle size] from Asahi Chemical Industry Co., Kawasaki-shi, Japan. The eluent was acetonitrile/methanol/1 M ammonium acetate (7:3:90, by vol.) buffer, pH 5.16, containing 0.27 mM EDTA. The flow rate was 0.5 ml/min. The porphyrinogens were detected electrochemically at +0.65 V with a LCA-15 detector from EDT Research, London N.W.10, U.K. For separation of heptacarboxylic porphyrinogen generated from [^{14}C]uroporphyrinogen III, the standard heptacarboxylic porphyrinogens were used as markers for the collection of the labelled compounds.

H.p.l.c. of hexacarboxylic porphyrin isomers

The hexacarboxylic porphyrin isomers were separated on a Hypersil-ODS column [250 mm × 5 mm (int.diam.)] with 16% (v/v) acetonitrile in 1 M ammonium acetate buffer, pH 5.16, as eluent (Lim et al., 1983). The porphyrins were detected fluorometrically at 405 nm (excitation) and 618 nm (emission).

H.p.l.c. of pentacarboxylic porphyrin isomers

The pentacarboxylic porphyrin isomers were also separated on the Hypersil-ODS column with 21% (v/v) acetonitrile in 1 M ammonium acetate buffer, pH 5.15, as mobile phase (Lim et al., 1983) and detected fluorometrically at excitation and emission wavelengths of 405 and 618 nm respectively.

Radioactivity counting

The radioactivity contents of the peaks corresponding to 7a, 7b, 7c and 7d derived from incubation of ^{14}C-uroporphyrinogen III were determined in Insta-Gel (10 ml) with a Wallac 1410 liquid-scintillation counter (Pharmacia, Wallacoy, Finland).

RESULTS AND DISCUSSION

The h.p.l.c. profile of the porphyrins formed by incubation of PBG with human red cell haemolysates is shown in Figure 2. All type III porphyrins were formed with 7 III as the major decarboxylation intermediate. Of the type I porphyrins, only uroporphyrin I was detectable. The time course of formation of intermediates and product is shown in Figure 3.

The effect of PBG concentration on intermediates and product formation is shown in Figure 4. Intermediates and product production rose rapidly and reached a plateau at about 50 μM PBG.

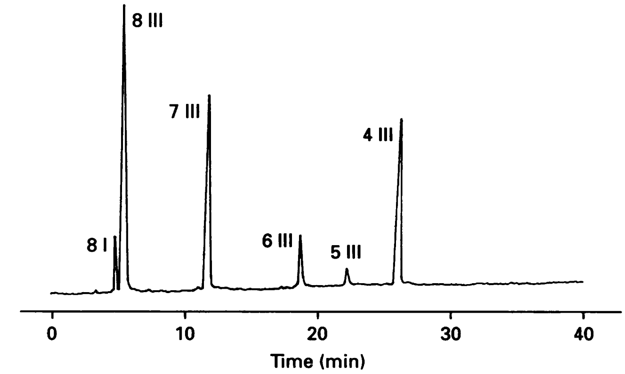

Figure 2 H.p.l.c. profile of the reaction products formed by incubation of PBG with red-cell haemolysates

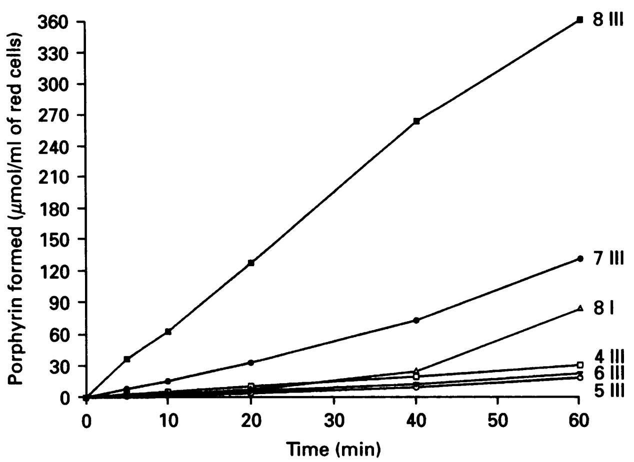

Figure 3 Time course of formation of uroporphyrin, decarboxylation intermediates and coproporphyrin following incubation of PBG (31.7 μM) with red-cell haemolysates

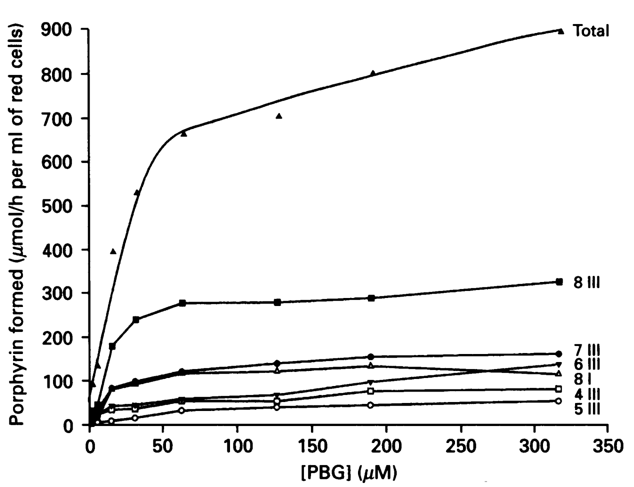

Figure 4 Effects of PBG concentration on the formation of uroporphyrin, decarboxylation intermediates and coproporphyrin

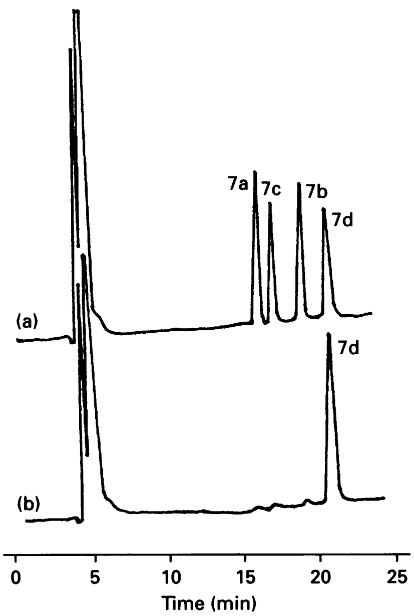

Figure 5 H.p.l.c. separation of heptacarboxylic porphyrinogen isomers from the enzyme incubation mixtures with (a) uroporphyrinogen III (3.7 μM) and (b) porphobilinogen (31.7 μM) as substrate respectively

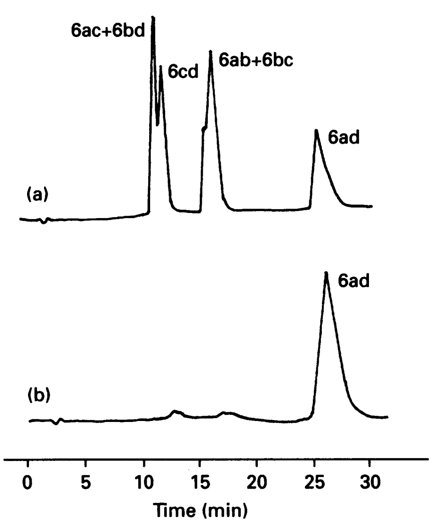

Figure 6 H.p.l.c. separation of hexacarboxylic porphyrin isomers from the incubation mixtures with (a) uroporphyrinogen (3.7 μM) and (b) porphobilinogen (31.7 μM) as substrate respectively

The separation of the heptacarboxylic porphyrinogens derived from the above reactions and from incubation of uroporphyrinogen III with red-cell haemolysates is shown in Figure 5. With PBG as substrate, 7d was the main isomer formed, and 7a, 7b and 7c were hardly detectable. The composition of the isomers was not affected by either the length of incubation (up to 60 min) or PBG concentration (3.17–317 μM). With uroporphyrinogen III as substrate, a mixture of the four heptacarboxylic porphyrinogen isomers was produced, as previously reported (Luo and Lim, 1990). These results clearly indicated that two different routes of decarboxylation are possible, depending on whether PBG or uroporphyrinogen III prepared by reduction (with 3 % sodium amalgam) of uroporphyrin III was used in enzyme reaction(s). The most obvious and significant differences in these two systems are the concentrations of uroporphyrinogen III present in the enzyme incubation system. The uroporphyrinogen III concentration generated by the combined action of hydroxymethylbilane synthase (EC 4.3.1.8) and uroporphyrinogen III synthase (EC 4.2.1.75) is much lower than that prepared by reduction of uroporphyrin III. Jones (1992) has suggested that, at the low, but constant, levels of uroporphyrinogen III generated by PBG, any subtle differences in the K_m for binding the four rings of uroporphyrinogen III to the enzyme would result in the selectivity of binding and consequently favouring an ordered decarboxylation sequence. A high initial concentration of uroporphyrinogen III could 'swamp' the system, and hence any subtle differences in K_m would be insignificant, resulting in random decarboxylation. The non-specificity of uroporphyrinogen decarboxylation from substrates such as uroporphyrinogen II and IV when presented in high concentration to the enzyme may be similarly explained.

The effect of uroporphyrinogen III concentrations on the isomer composition was investigated with [^{14}C]uroporphyrinogen III as substrate. At 0.01 μM substrate concentration, about 60 % of the heptacarboxylic porphyrinogen formed was 7d. The proportion of 7d would probably be higher at substrate con-

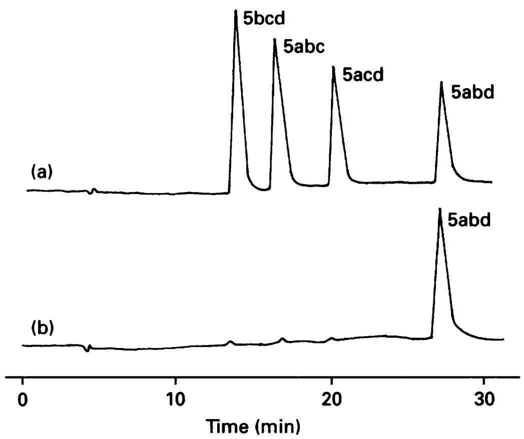

Figure 7 H.p.l.c. separation of pentacarboxylic porphyrins from the incubation mixtures with (a) uroporphyrinogen III (3.7 μM) and (b) porphobilinogen (31.7 μM) as substrate respectively

centrations lower than 0.01 μM. However, heptacarboxylic porphyrinogens were unsufficiently measurable at such low substrate concentrations to allow accurate quantification. At a uroporphyrinogen III concentration of 0.05 μM, the isomer composition was 17% 7a, 17% 7b, 16% 7c and 40% 7d. We have already shown that, at higher uroporphyrinogen III concentrations, 7a, 7b, 7c and 7d were formed in virtually equal quantities (Luo and Lim, 1990). It is therefore obvious that the order of decarboxylation is substrate-concentration-dependent, with low substrate concentration favouring the selective decarboxylation of the ring-D acetate group.

At higher PBG concentrations ($> 50\ \mu$M) and longer incubation times (60 min), relatively high levels of uroporphyrinogen III were observed (Figures 3 and 4). This, however, did not lead to random decarboxylation. A possible explanation is that the uroporphyrinogen III–uroporphyrinogen III synthase complex derived from PBG in the process of being transferred to uroporphyrinogen decarboxylase for the decarboxylation reaction did not equilibrate fast enough with the uroporphyrinogen III released in the incubation mixture.

To determine whether the presence of PBG and/or uroporphyrinogen I in the incubation mixture affects the proportion of each heptacarboxylic porphyrinogen isomer formed, these compounds were added individually and as a mixture to the red-cell haemolysate system preincubated with PBG or uroporphyrinogen III. No alteration in isomer composition was observed.

The h.p.l.c. separation of the hexacarboxylic porphyrins formed by incubation of PBG is shown in Figures 6(a) and 6(b), respectively. A mixture of isomeric type III hexacarboxylic porphyrins was formed by incubation of uroporphyrinogen III. This is not surprising, since decarboxylation of the mixture of 7a, 7b, 7c and 7d produced in the first step of enzyme reaction would be expected to give rise to a mixture of hexacarboxylic porphyrins. Incubation of PBG, however, gave predominantly the 6ad isomer. As the main heptacarboxylic porphyrinogen formed was 7d in this case, the formation of 6ad indicated selective decarboxylation of the ring-A acetate group of 7d, i.e. in the clockwise direction.

The random enzymic decarboxylation of uroporphyrinogen III to a mixture of isomeric hepta- and hexa-carboxylic porphyrinogens also led to the formation of all four possible type III pentacarboxylic porphyrins (Figure 7a). The pentacarboxylic porphyrins derived from incubation of PBG, on the other hand, was virtually all of the 5abd isomer (Figure 7b). There was again a selective decarboxylation of the acetate group in the clockwise direction, i.e. the acetate group on the ring **B** of 6ad, to give 5abd. The predominant production of 7d, 6ad and 5abd following incubation of PBG with red-cell haemolysates thus provided direct experimental confirmation of a clockwise decarboxylation sequence originally proposed by Jackson et al. (1976). Lash (1979, 1991) has reported that, with exogenous 7d and 6ad as substrates, a mixture of isomeric decarboxylation intermediates was formed, with no indication of a clockwise mechanism. We therefore concluded that two different decarboxylation routes were possible, depending on whether uroporphyrinogen III was supplied exogenously in excess, i.e. in high concentration, or derived endogenously from PBG, i.e. at low concentration; the former was random and the latter was clockwise and orderly. It also follows that, under normal physiological conditions, enzymic decarboxylation is most probably orderly and clockwise.

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