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Metabolism of pentacarboxylate porphyrinogens by highly purified human coproporphyrinogen oxidase: Further evidence for the existence of an abnormal pathway for heme biosynthesis☆

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Abstract—An abnormal series of porphyrin tetracarboxylic acids known as the isocoproporphyrins, are commonly excreted by patients suffering from the disease porphyria cutanea tarda (PCT). These porphyrins appear to arise by bacterial degradation of dehydroisocoproporphyrinogen that is generated by the premature metabolism of the normal pentacarboxylate intermediate (5dab) by coproporphyrinogen oxidase (copro'gen oxidase). This porphyrinogen can be further metabolized by uroporphyrinogen decarboxylase to give harderoporphyrinogen, one of the usual intermediates in heme biosynthesis. Therefore, it is possible that some of the heme formed under abnormal conditions may originate from the 'isocopro-type' porphyrinogen intermediate. In order to investigate the feasibility of alternative pathways for heme biosynthesis, the four type III pentacarboxylate isomeric porphyrinogens were incubated with purified, cloned human copro'gen oxidase at 37 °C with various substrate concentrations under initial velocity conditions. Of the four isomers, only 5dab was a substrate for copro'gen oxidase and this gave dehydroisocoproporphyrin. The structure of the related porphyrin tetramethyl ester was confirmed by proton NMR spectroscopy and mass spectrometry. The K_m value for proto'gen-IX formation from copro'gen, an indicator of molecular recognition, was similar to the K_m value for monovinyl product formation with 5dab, although copro'gen-III has an approximately twofold higher K_{cat} value. Although 5dab is a slightly poorer substrate than copro'gen-III, these results support the hypothesis that an abnormal route for heme biosynthesis is possible in humans suffering from PCT or related syndromes such as hexachlorobenzene poisoning.
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1. Introduction

In mammals, the heme biosynthetic pathway takes place via eight enzyme-mediated steps starting from glycine and succinyl CoA, although in plants and bacteria the first intermediate, δ-aminolevulinic acid, is derived from glutamate.[1,2] The first macrocyclic intermediate in the pathway, uroporphyrinogen-III (uro'gen-III; **1**), is the precursor to the vitamin B_{12} and related methylated tetrapyrroles such as siroheme, as well as the hemes and chlorophylls.[1,2] The cytosolic enzyme uro'gen decarboxylase converts octacarboxylic acid **1** to the tetracarboxylic acid coproporphyrinogen-III (copro'gen-III; **2**) by mediating the decarboxylation of four acetate side chains to give methyl units (Scheme 1).[3,4] At this stage, **2** is transferred into the mitochondria and undergoes two oxidative decarboxylations by the enzyme copro'gen oxidase to give proto'gen-IX (**3**).[5] Further oxidation of the hexahydroporphyrin **3** by proto'gen oxidase gives protoporphyrin-IX, and subsequent iron(II) insertion by ferrochelatase leads to heme *b*. Protoporphyrin-IX is the precursor to many other heme type pigments, and is also a key intermediate in the formation of the chlorophylls in photosynthetic organisms.[1,2]

In principle, uro'gen-III could be converted into copro'gen-III by 24 different pathways involving up to 14 different intermediates.[3,4,6] Depending upon whether the initial acetate group undergoes decarboxylation on ring A, B, C, or D, four heptacarboxylate intermediates (hepta's) may be formed (Chart 1). These, in turn, could each be transformed at one of three different sites to form a total of six different hexacarboxylate porphyrinogens (hexa's), and these could then be taken on via two pathways each to give four pentacarboxylate species (penta's). The four penta's only have one remaining

Keywords: Porphyrinogens; Isocoproporphyrins; Heme biosynthesis; Coproporphyrinogen oxidase; Porphyria.

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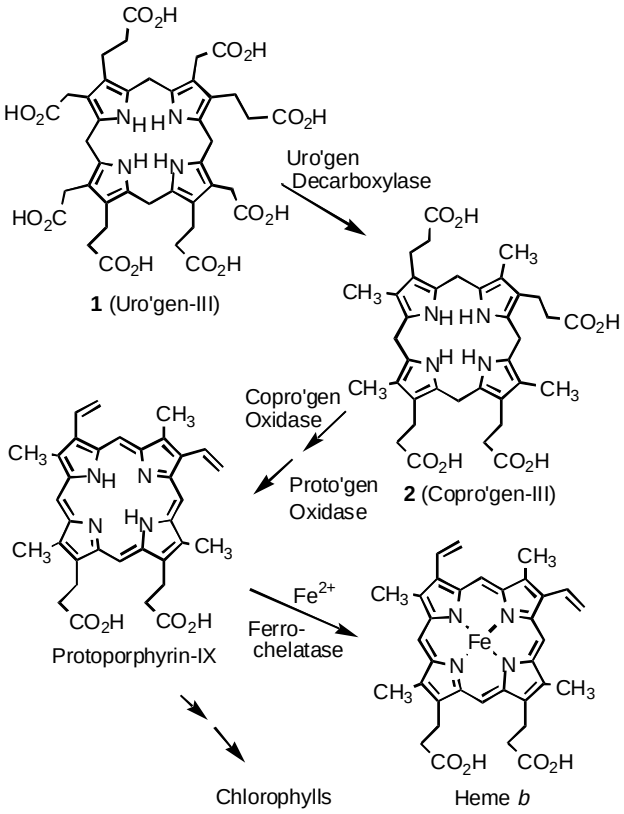


Scheme 1. Biosynthesis of heme from uroporphyrinogen-III.

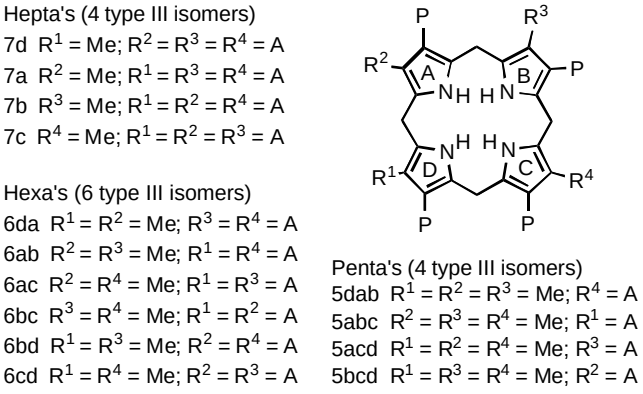
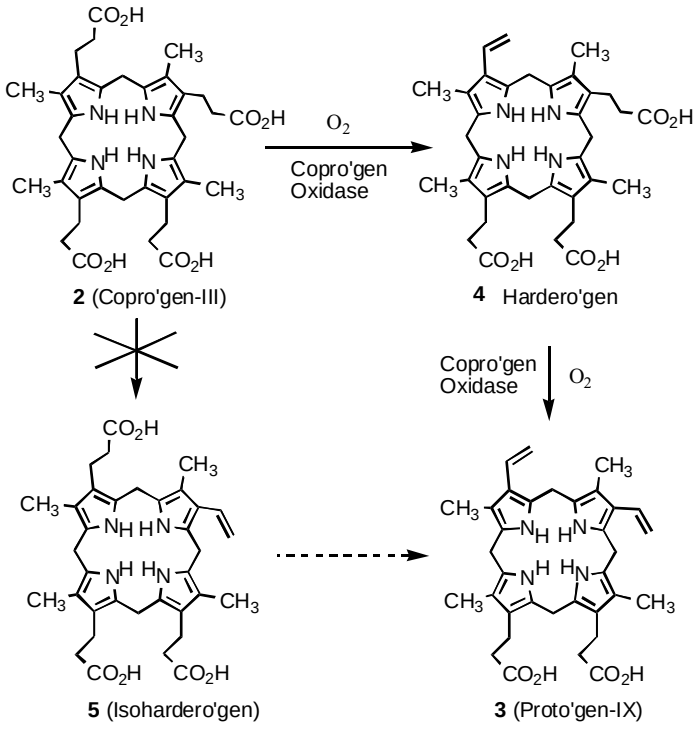


Chart 1. The 14 possible intermediates between uro'gen-III and copro'gen-III. The letter code designations refer to the rings (labeled A–D in the porphyrin structure, but assigned in the structure codes with the lower case letters a, b, c, and d) on which the acetate units have been decarboxylated, while the numbers correspond to the number of remaining carboxylate groups.[3] Under physiological conditions, the intermediates are 7d, 6da, and 5dab. Abbreviations for substituents: A = CH_2CO_2H; P = $CH_2CH_2CO_2H$.

acetate moiety and the final step for each would converge to give copro'gen-III. Early studies demonstrated that the process was sequential and the related porphyrin fractions corresponding to hepta's, hexa's, and penta's can be identified.[7] In the 1970s, these porphyrin fractions were isolated from rats poisoned with hexachlorobenzene and the corresponding methyl esters were shown to be 7d, 6da, and 5dab, respectively, by total synthesis[8–10] and proton NMR spectroscopy.[3,11] In addition, 7d was identified as the major hepta component from a patient suffering from porphyria cutanea tarda (PCT)[11] and in incubations of porphobilinogen with chicken red cell hemolysates containing a high concentration of sodium chloride.[10] On the basis of these data, Jackson postulated that uro'gen-III was transformed into copro'gen-III by a selective route, where the D ring undergoes the first decarboxylation followed by degradation at ring A, then B, finally C (the 'clockwise decarboxylation' hypothesis).[3] However, HPLC studies showed that mixtures of hepta's, hexa's, and penta's were present in normal urine,[12,13] and to add to the difficulty, were also formed in incubations of uro'gen-III with crude preparations of uro'gen decarboxylase.[6,14] However, at very low substrate concentrations, uro'gen-III shows a higher preference for 7d over the remaining hepta's,[15] and the current consensus is that while a degree of random processing may occur under some circumstances, the clockwise decarboxylation pathway operates under physiological conditions.[6,15]

Copro'gen-III is converted into proto'gen-IX (Scheme 2) via the tricarboxylate intermediate harderoporphyrinogen (hardero'gen; **4**), rather than the alternative species isoharderoporphyrinogen (**5**).[16,17] There is no ambiguity about whether the A or B ring is processed first for this enzyme as **5** is a very poor substrate for copro'gen oxidase[18] and only porphyrins derived from **4** are observed in enzyme incubation studies.[16,17] Harderoporphyrin was first isolated and characterized from the harderian glands of rodents,[19] but has been identified in feces and other natural materials.[20]

In 1972, Elder reported the isolation of a series of tetracarboxylate porphyrins **6a–d** (Chart 2) from porphyric patients and rats poisoned with hexachlorobenzene.[21] The ethyl-substituted compound has the same molecular weight as coproporphyrin-III, and, for this reason, this metabolite was named isocoproporphyrin.[21,22] The structure of **6a** has been rigorously demonstrated by degradation and proton NMR studies using europium shift reagents,[23] as well as by total synthesis.[24–26] It was proposed that these unusual compounds are all derived from the 3-vinyl species, dehydroisocoproporphyrin **6b**, or the related porphyrinogen **7b**, by bacterial degradation in the gut.[21,22] Indeed, degradation of the side chains for protoporphyrin-IX commonly gives rise to related porphyrins such as deuteroporphyrin-IX, pemptoporphyrin, mesoporphyrin-IX, etc.[20] Evidence for a fifth member of the series, the 3-acetylporphyrin **6e**, has also been presented.[27] The presence of an acetate group on ring C suggests that 5dab has been prematurely metabolized by copro'gen oxidase. PCT and hexachlorobenzene poisoning are both associated with an accumulation of hepta-, hexa-, and pentacarboxylate intermediates, and this may allow leakage of 5dab into the mitochondria where copro'gen oxidase recognizes this species and converts it into **7b**.[28,29] It is possible that **7b** could be further transformed into protoporphyrin-IX, and this species represents a potential intermediate in an abnormal 'isocopro' pathway for heme biosynthesis (Scheme 3).[29] Pentacarboxylate porphyrinogen 5dab



Scheme 2. Conversion of copro'gen-III to proto'gen-IX.

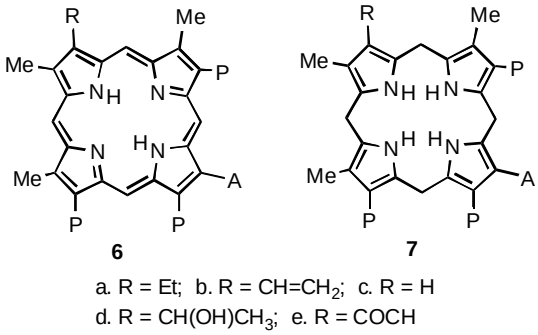
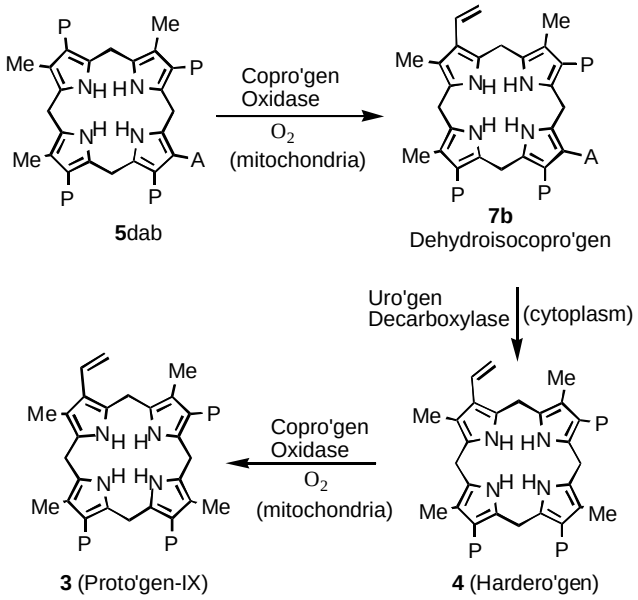


Chart 2. Structures of the isocoproporphyrin series and the related porphyrinogens. It should be noted that porphyrins **6** were synthesized and characterized as the corresponding tetramethyl esters. Abbreviations: A = CH_2CO_2H; P = $CH_2CH_2CO_2H$.

has been shown to be converted to **7b** by rat liver preparations of copro'gen oxidase, but further conversion to a divinyl product is not observed.[28] Synthetic isocoproporphyrinogen (**7a**) is also not a substrate for the rat liver enzyme. However, **7a**, **7b**, and **7c** have been shown to be metabolized by the uro'gen decarboxylase present in chicken red cell hemolysates to give hardero'gen or related tricarboxylate porphyrinogens, and these can then be further processed by copro'gen oxidase.[29] These data show that an abnormal pathway is possible for proto'gen-IX production following the sequence 5dab to **7b** to hardero'gen to proto'gen-IX (Scheme 3), although the intermediates would have to be shuttled back and forth between the mitochondria and cytoplasm at each step. The formation of dehydroisocoproporphyrinogen by mutants of *Saccharomyces cerevisiae* that are partially deficient in uro'gen



Scheme 3. Proposed abnormal pathway for heme biosynthesis.

decarboxylase activity suggests that this pathway could operate in yeast as well.[30]

The observations noted above, together with extensive studies using substrate analogues, has allowed the development of a model for substrate binding in copro'gen oxidase (Fig. 1). A sequence of substituents R Me–P Me–P, where R is a small nonpolar group such as H, Me, Et, or vinyl, and P = propionate, appears to be needed for substrate binding and oxidative decarboxylation of the first propionate residue.[31,32] Changing

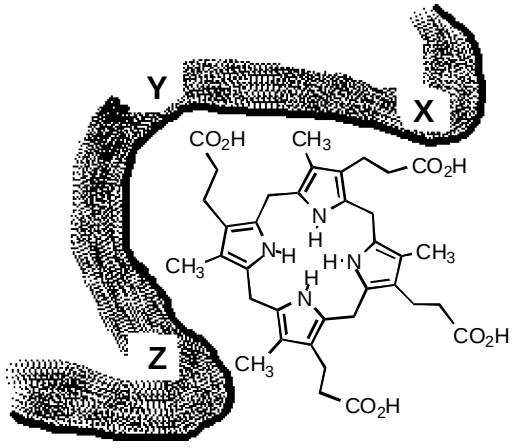


Figure 1. Proposed model for the active site of copro'gen oxidase.

either of the propionate side chains to butyrate, $CH(CH_3)CH_2CO_2H$, or $CH_2CH(CH_3)CO_2H$ leads, at best, to much reduced activity.[33] The proposed model (Fig. 1) identifies three significant regions within the active site for copro'gen oxidase.[5,32,34–36] Site X recognizes the second propionate group, probably via a salt bridge interaction, Y represents the catalytic site, while region Z can only tolerate small nonpolar groupings. Although this model has been very successful in predicting the substrate specificity for copro'gen oxidase,[34–36] it is not clear what types of minor changes can be tolerated in the peripheral sequence. R cannot be a propionate group, but a $CH = CH–CH_3$ unit does appear to fit while $C(CH_3) = CH_2$ is poorly tolerated.[33] The observation that **7a** or **7b** are not substrates for rat liver copro'gen oxidase shows that the second methyl group in the sequence cannot be replaced by an acetate unit, but it is not clear whether acetate groups could be present in place of the first methyl group or instead of the usual nonpolar groupings for R. As four penta's can be formed from uro'gen-III,[3,4] alternative 'neocopro' pathways for heme biosynthesis could exist, and to our knowledge this possibility has never been tested. In addition, studies of this type using purified human copro'gen oxidase have not been conducted previously. In this paper, we report the first studies of the abnormal 'isocopro' pathway using cloned human copro'gen oxidase and investigate the metabolism of all four type III pentacarboxylate porphyrinogens.

2. Results and discussion

Highly purified enzyme (12 mg/L of cell culture, single band by SDS–PAGE, MW of 37,000) was obtained for these studies (data not shown). Pentacarboxylate porphyrinogens 5dab, 5abc, 5abd, and 5acd were prepared by hydrolysis of the corresponding porphyrin methyl esters and reduction with 3% sodium amalgam under standard conditions.[5] The pentacarboxylate porphyrins were previously synthesized via a,c-biladiene intermediates.[4] Following incubation of the porphyrinogens with the cloned human enzyme preparations, the metabolites were analyzed as the corresponding porphyrin methyl esters using normal phase HPLC. Repeated experiments demonstrated that only 5dab was a substrate for copro'gen oxidase, and the three isomeric type III porphyrinogens showed no indication of product formation. These data indicate that acetate moieties cannot be tolerated in place of group R at site Z in the active site model, or in place of the first methyl unit in the sequence. On the other hand, 5dab proved to be a very good substrate giving dehydroisocoproporphyrinogen **7b** as the only product, and in agreement with earlier investigations no further metabolism of the B ring propionate residue was observed.

In order to further assess how good a substrate 5dab is for cloned human copro'gen oxidase, more detailed kinetic studies were conducted. Figure 2a shows the apparent initial velocity (v_0) for the accumulation of total, divinyl (**3**), and monovinyl (**4**) products after incubation of 7.5 μg of the purified enzyme with the authentic substrate copro'gen-III (1 μM). Figure 2b shows the monovinyl product **7b** formed from 5dab (1 μM). Total product was calculated as the sum of the monovinyl and divinyl products using copro'gen-III. These data are from three replicate incubations, and the range of high and low values about the mean was 5%. The equations for the apparent linear portion of the lines (from 0 to 2 min for copro'gen-III and 5 min for 5dab) were evaluated by linear regression analysis. The initial velocity for copro'gen-III was more than twofold faster than that of 5dab (19.8/min and 8.2/min, respectively). Figure 3a shows v_0 (nmol product/min/pmol enzyme) as a function of substrate concentration from 0.01 to 6.1 μM using copro'gen-III as substrate. Figure 3b shows v_0 as a function of 5dab substrate concentration from 0.01 to 6.6 μM. The kinetic constants (K_m, K_{cat}, and K_{cat}/K_m), using both substrates, are shown in Table 1. When comparing total product, copro'gen-III has about a twofold lower K_m than 5dab, approximately a twofold higher K_{cat}, and about a fourfold higher K_{cat}/K_m ratio. Even though the values for the same kinetic constants are within an order of magnitude of each other, the K_{cat}/K_m values, a measure of catalytic efficiency, indicate that the sequential oxidative decarboxylations of the C-III substrate are fourfold more efficient than the single oxidative decarboxylation of 5dab. Hence, even though the presence of an acetate group on ring C does not significantly inhibit metabolism, it still appears to have a small detrimental influence.

In order to rigorously confirm that dehydroisocoproporphyrin **6b** had in fact been generated from 5dab, a larger scale experiment was carried out and the isolated porphyrin product was characterized by proton NMR spectroscopy and mass spectrometry. The proton NMR spectrum of the isolated product (Fig. 4a) shows the presence of seven methyl groups between 3.6 and 3.8 ppm corresponding to the four methyl esters and the three porphyrin methyl groups. The vinyl moiety was evident from the three doublet of doublets at 6.17, 6.36, and 8.28 ppm, while the three propionate side chains afforded two 6H multiplets at 3.3 and 4.5 ppm, and the methylene component of the acetate group gave a 2H singlet at 5.1 ppm. Finally, the internal NHs were

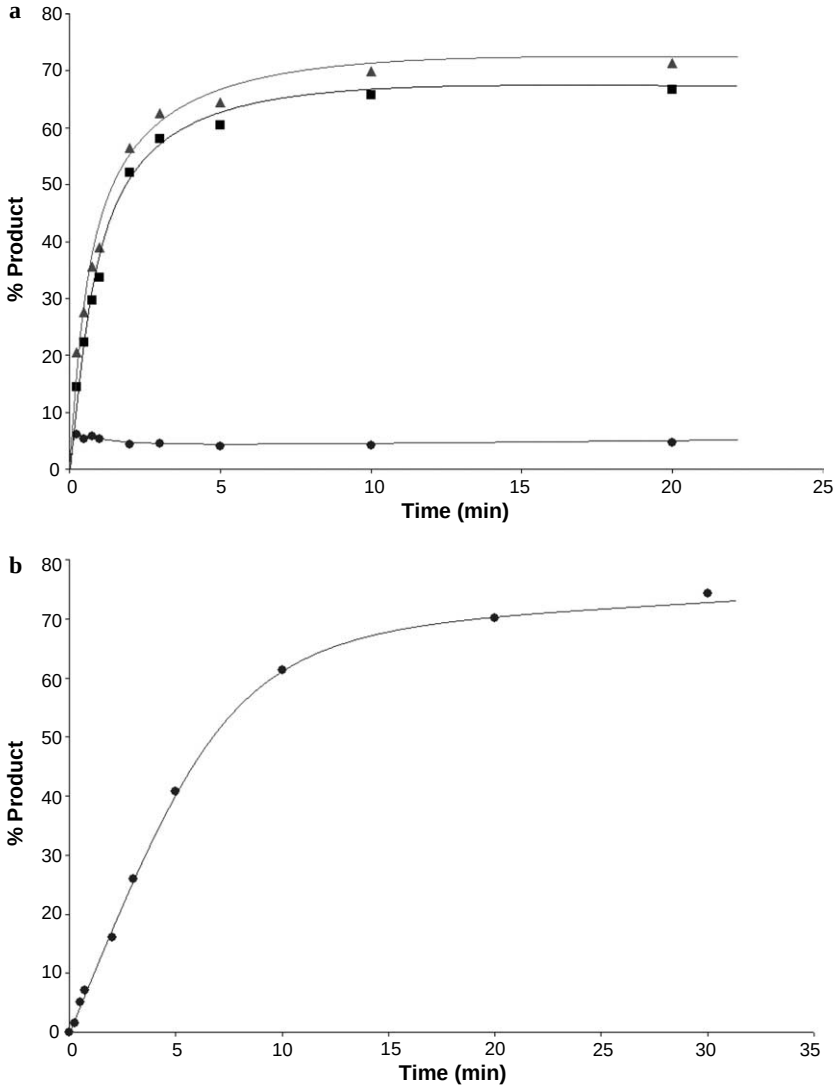


Figure 2. Time course experiments for copro'gen-III (a) and 5dab (b) showing the the percent product formation versus time. For (a), triangles represent total % product accumulation, squares represent % divinyl product accumulation, and circles represent % monovinyl product formation from copro'gen-III. For (b), circles indicate the % monovinyl product formed from 5dab. All values are the mean of three replicate incubations. The range of high and low values about the mean was 5%.

evident as a 2H singlet at −3.7 ppm, while the *meso*-protons gave three singlets in the ratio of 2:1:1 near 10.2 ppm. In the original structure determination of isocoproporphyrin, europium shift reagents were used to show how the ester groups were arranged around the periphery of the macrocycle. When two ester groups flank a given *meso*-proton, the shift reagent associates relatively strongly at that site causing a significant downfield shift to the nearby *meso*-resonances. Addition of $Eu(fod)_3$ to our NMR solution in $CDCl_3$ showed that two of the *meso*-protons were shifted downfield and significantly broadened, while the other two resonances were essentially unaffected (Fig. 4b). This is exactly the result expected as two of the *meso*-protons are surrounded by two ester units in **6b**, while the other two have no ester moieties nearby. FAB MS provided further confirmation of the structure and gave the expected $[M+H]^+$ ion at *m*/*z* 709.

3. Conclusion

The metabolism of 5dab by purified human copro'gen oxidase has been demonstrated, but the other three type III pentacarboxylate porphyrinogens are not metabolized by this enzyme. Porphyrinogen 5dab appears to bind to the active site with good affinity, having a K_m of 1.3 μM, which is comparable to the K_m of copro'gen-III, which is 0.54 μM, and the kinetic values show that this pentacarboxylate substrate is a good substrate when considering the turnover rate (K_{cat}). Hence, the feasibility of an abnormal 'isocopro' pathway for heme biosynthesis is supported by these data, while the possibility of other 'neocopro' pathways can now be dismissed.

In disease states where 5dab is released prematurely from uro'gen oxidase, it can compete with the normal

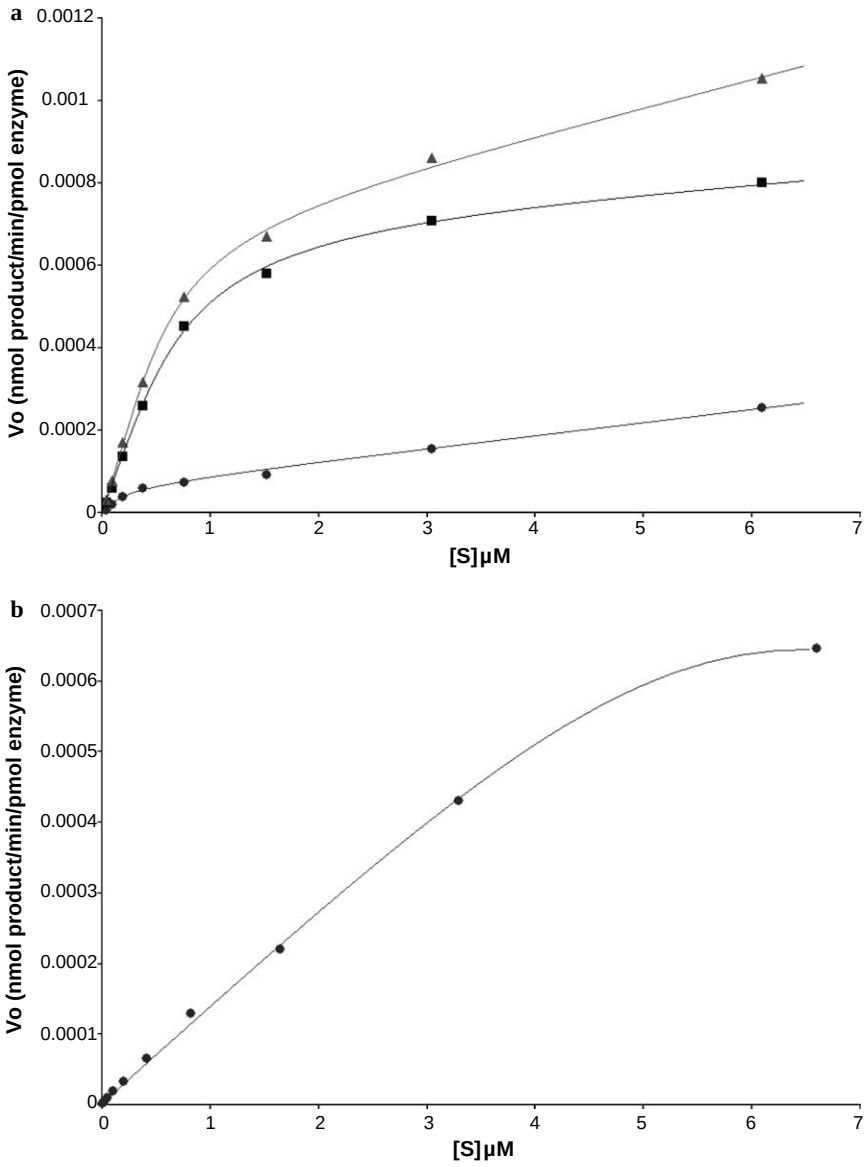


Figure 3. (a) Initial velocity curve as a function of substrate concentration with copro'gen-III. (b) Substrate concentration curve with 5dab. All values are the mean of three replicate incubations. The range of high and low values about the mean was 5%. See Figure 2 for key.

Table 1. Kinetic constants of copro'gen oxidase for monovinyl, divinyl, and total product accumulated

	Substrate	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m
Monovinyl product	C-III	0.97	0.20	0.21
	5dab	1.3	0.54	0.42
Divinyl product	C-III	0.55	0.75	1.4
Total product	C-III	0.54	0.97	1.8
	5dab	1.3	0.54	0.42

substrate **2** for the active site of copro'gen oxidase and generate dehydroisocoproporphyrinogen. However, further metabolism to proto'gen-IX can only occur if the porphyrinogen migrates back into the cytoplasm to be acted upon by uro'gen decarboxylase to form harder-o'gen, followed by transport back into the mitochondria so that the porphyrinogen can be taken on to proto'gen-IX and ultimately to heme *b*. This convoluted process greatly increases the likelihood that spontaneous oxidation to the porphyrin form will occur. As the aromatic forms are not recognized by either of the enzymes, these abnormal metabolites would accumulate, exacerbating symptoms such as light sensitivity for porphyric patients.

4. Experimental

4.1. Isolation and purification of copro'gen oxidase

Escherichia coli, containing the gene for the human enzyme, were grown overnight at 37 °C with shaking at 250 rpm, in 1-L cultures using a medium consisting of 10 g tryptone (Fischer Chemical Company), 5 g yeast extract (Sigma Chemical Company), and 10 g NaCl per liter nanopure water. Cells were isolated by

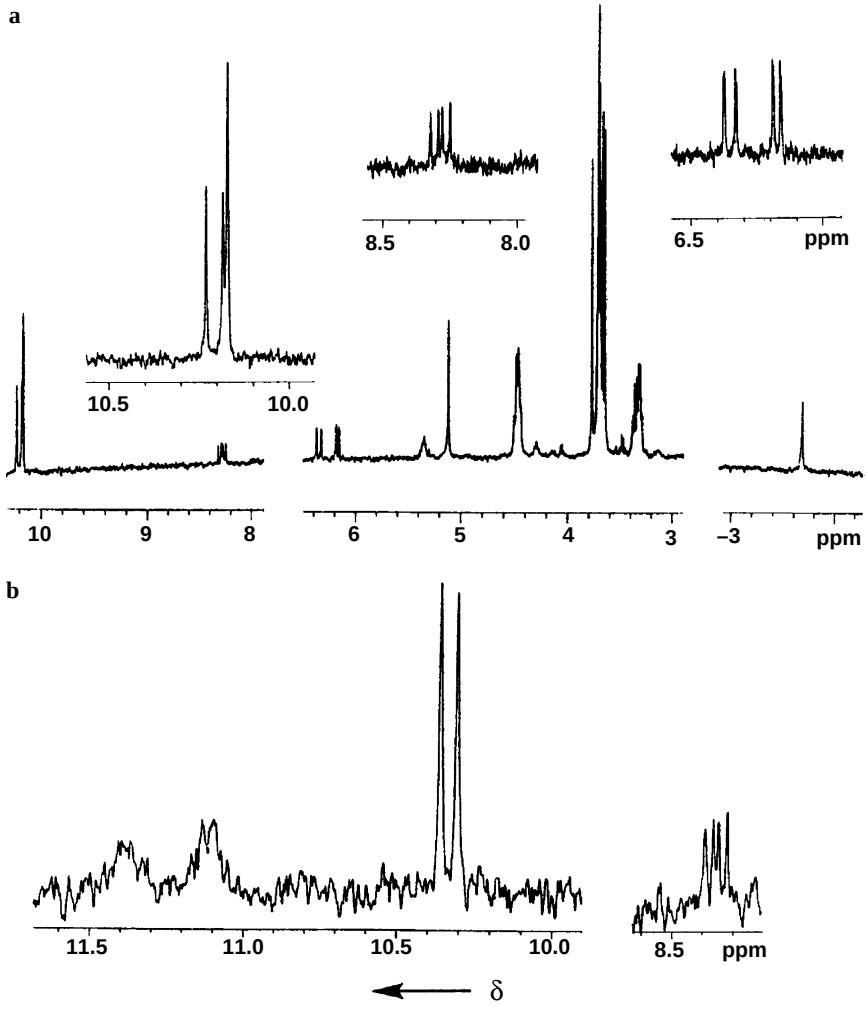


Figure 4. (a) 400 MHz proton NMR spectrum in $CDCl_3$ of dehydroisocoproporphyrin tetramethyl ester isolated from incubations of 5dab with copro'gen oxidase. (b) Downfield region for the same sample in the presence of the europium shift reagent $Eu(fod)_3$. Two of the four *meso*-protons were shifted downfield producing broadened resonances while the other two were essentially unaffected.

centrifugation and lysed using the French hydraulic pressure cell (Sim-Aminco Spectronic Instruments). The enzyme, which has the $6\times$ histidine tag, was isolated by the Ni^{2+} affinity procedure of Medlock and Dailey.[37] Protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli method[38] to test the purity and apparent molecular weight of the enzyme. The Bradford Protein Assay[39] was used to evaluate the concentration of the enzyme and bovine serum albumin (BSA) was used as the standard.

4.2. Enzyme assay

Using the micromethod of Jones et al.,[40] copro'gen-III, 5dab, 5abc, 5bcd, or 5cda were incubated with the copro'gen oxidase (300 μL of 25 μg/mL) at 0, 0.25, 0.5, 0.75, 1, 2, 3, 5, 10, 20, or 30 min for one set of experiments using 1 μM substrate. In another set of experiments, substrate concentration was varied from 0.01 to 6.1 μM, while holding the incubation time constant at 1 min for copro'gen-III, and from 0.01 to 6.6 μM for an incubation time of 3 min for 5dab. The reactions were stopped with addition of 3/7 (v/v) acetic acid/ethyl acetate followed by extraction and methyl esterification overnight. In all experiments, a zero incubation control (addition of acetic acid/ethyl acetate before addition of substrate) was also performed for comparison. Following neutralization and extraction of the porphyrin methyl esters, they were analyzed using high pressure liquid chromatography (HPLC; Beckman System Gold). A normal phase column (Beckman Silica 5 μ, 4.6 mm × 25 cm) was used with a solvent of 35/65 (v/v) ethyl acetate/cyclohexane for C-III and 50/50 (v/v) for the pentacarboxylates at a flow rate of 1.3 mL/min; elutes were evaluated spectrophotometrically at a wavelength of 404 nm. Data were analyzed using the Gold Nouveau Software and reported as percent product or subsequently converted to the units of Medlock and Dailey.[37] Incubations with the other three pentacarboxylate porphyrinogens showed no detectable product under any conditions tested.

4.3. Isolation of dehydroisocoproporphyrin tetramethyl ester

In order to collect sufficient product for proton NMR characterization, a 1 L scale incubation was performed.

Cloned copro'gen oxidase (25 mg) was isolated from 2 L of *E. coli* cells and diluted in 250 mM imidazole buffer (pH 7.0) to give 1 L. This preparation was incubated with 1 mg of porphyrinogen 5dab for 1 h at 37 °C with gentle shaking. The incubation was terminated by the addition of 2.85 L 3/7 (v/v) acetic acid/ethyl acetate and extracted as described previously. Following esterification with 5% sulfuric acid–methanol at room temperature for 16 h, HPLC showed that the extract contained 80% product. Purification by flash chromatography on silica eluting with 3/7 ethyl acetate/toluene gave the 3-vinylporphyrin. ^{1}H NMR (400 MHz, $CDCl_3$): δ −3.67 (2H, s), 3.29–3.38 (6H, m), 3.65 (3H, s), 3.67 (3H, s), 3.69 (3H, s), 3.70 (3H, s), 3.71 (6H, s), 3.77 (3H, s), 4.42–4.50 (6H, m), 5.12 (2H, s), 6.17 (1H, dd, J = 1.5, 11 Hz), 6.36 (1H, dd, J = 1.5, 17 Hz), 8.28 (1H, dd, J = 11, 17 Hz), 10.17 (2H, s), 10.18 (1H, s), 10.23 (1H, s). HRMS (FAB): Calcd for $C_{40}H_{44}N_4O_8$ + H: 709.3237. Found: 709.3236.

Acknowledgments

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