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- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

# Kinetic evaluation of human cloned coproporphyrinogen oxidase using a ring isomer of the natural substrate

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## Summary

**Background:**

The enzyme coproporphyrinogen oxidase (copro'gen oxidase) converts coproporphyrinogen-III (C-III) to protoporphyrinogen-IX via an intermediary monovinyl porphyrinogen. The A ring isomer coproporphyrinogen-IV (C-IV) has previously been shown to be a substrate for copro'gen oxidase derived from avian erythrocytes. In contrast to the authentic substrate (C-III) where only a small amount of the monovinyl intermediate is detected, C-IV gives rise to a monovinyl intermediate that accumulates before being converted to an isomer of protoporphyrinogen-IX. No kinetic studies have been carried out using the purified human copro'gen oxidase to evaluate its ability to process both the authentic substrate as well as analogs.

**Materials/Methods:**

Therefore, purified, cloned human copro'gen oxidase was incubated with C-III or C-IV at 37°C with various substrate concentrations (from 0.005 µM to 3.5 µM). The Km (an indication of molecular recognition) and Kcat (turnover number) values were determined.

**Results:**

The Km value for total product formation was about the same with either C-III or C-IV indicating the same molecular recognition. However, the catalytic efficiency (Kcat/Km) of the enzyme for total product formation was not more than two fold higher using C-III relative to C-IV.

**Conclusions:**

Since the Km values are about the same for either substrate and the total Kcat/Km values are within two fold of each other, this could correlate with the increase of severity of porphyrias with monovinyl accumulation. The ability of the increased levels of C-IV to compete with the authentic substrate has important implications for clinical porphyrias.

**key words:**

**coproporphyrinogen oxidase • porphyria • Km • Kcat • substrate analog**

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## BACKGROUND

Porphyrias are a group of clinical disorders, either genetic or acquired, that are characterized by accumulation of one or more type of porphyrins in tissues as well as higher levels of excretion in the urine and/or feces. These patients are generally characterized with mild to severe mental retardation, photosensitivity, and problems in the liver and/or bone marrow. Thus, understanding the enzymes involved in the heme biosynthesis pathway and their ability to process both the authentic substrates and analogs of these substrates is important in helping to develop clinical therapies for porphyrias. Defects in the various enzymes of the synthetic pathway have been correlated with various classes of porphyrias but much work yet remains.

Coproporphyrinogen oxidase (copro'gen oxidase; EC 1.3.3.3) is the sixth enzyme in the biosynthetic pathway for the production of heme. Coproporphyrinogen-III (C-III), the authentic substrate for the enzyme copro'gen oxidase, undergoes two sequential oxidative decarboxylations at the A and B ring propionates to produce first a monovinyl product and then a divinyl product (Figure 1A).

Harderoporphyria is a disorder often characterized by an accumulation of monovinyl porphyrins in the body and considered to be related to mutations in the gene encoding copro'gen oxidase. In addition, C-III and its isomer coproporphyrinogen-IV (C-IV) have been isolated from these types of patients [1]. Yet, C-IV also has been reported in the urine of patients with a defect in the enzyme 5-aminolevulinic acid dehydratase [2]. Kühnel et al. [1] also reported the presence of C-IV in the urine of normal people, although in much lower amounts.

Modified porphyrinogens have been used to probe the active site of copro'gen oxidase. C-IV was first found to be a substrate for ox-liver copro'gen oxidase by Porra and Falk in 1964 [3], and was subsequently shown to be converted to protoporphyrinogen-XIII via a monovinyl intermediate (Figure 1B). Jackson et al., using crude enzyme preparations from chicken red blood cells, reported that the monovinyl intermediate from C-IV accumulated to about 40% total porphyrinogen at intermediary incubation times, but was eventually converted to the divinyl product [4]. This contrasts to time study experiments with authentic C-III where accumulation of the monovinyl species, harderoporphyrogen, was less than 10%. In addition, Buldain et al. showed that incubations of C-IV with crude copro'gen oxidase preparations from duck blood, chicken blood and beef liver gave divinyl product at a rate of only 10% that of C-III [5]. These studies also showed that the type isomers coproporphyrinogen-I and coproporphyrinogen-II were not substrates for copro'gen oxidase. On the bases of these and other investigations with substrate analogs [6-9], Lash et al. proposed a substrate recognition model for the enzyme [10]. In this model, there are requirements for the correct sequence of the peripheral substituents around the macrocycle. In order to provide the correct "fit", the sequence R Me-P Me-P is required, where Me =  $-\text{CH}_3$ , P =  $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$  and R is a small nonpolar grouping such as Me, ethyl, or vinyl. When C-III is metabolized, a vinyl group is generated at ring A. The model predicts that the substrate remains in the active site and can rotate through  $90^\circ$  and present the active site

with the correct sequence of substituents for the second "B ring" propionate group to undergo oxidative decarboxylation. The Lash et al. paper also models the active site with C-IV leaving the active site and re-entering prior to the second oxidative decarboxylation [11]. This isomer of the authentic substrate has the sequence of the methyl and propionate groups on ring A switched, giving the molecule a plane of symmetry that is absent in C-III, and also providing two sequences of substituents that can be recognized at the active site. However, the active site model predicts that once the first oxidative decarboxylation has occurred, the second propionate side chain cannot be correctly orientated until the intermediate has dissociated from the binding pocket, flipped over, and reentered into the active site. This prediction provided a tentative explanation for the differences in the observed kinetics using both substrates by Jackson et al. [4]. Since C-IV can be processed as a substrate by normal (wildtype) copro'gen oxidase, this would result in a divinyl product with a concomitant accumulation of a monovinyl species. We speculate that the accumulation of both the mono- and divinyl products from C-IV in the mitochondria could then be correlated with clinical symptoms of the patients with harderoporphyria, resulting from the defect in a previous enzyme rather than in the copro'gen oxidase enzyme. However, the active site model was designed from results observed using crude preparations of copro'gen oxidase from avian erythrocytes. As yet there have been no reports on the kinetics for C-IV metabolism using highly purified human enzyme preparations. In this work, experiments were performed using purified preparations of the cloned human enzyme. The kinetic constants ( $K_m$ ,  $K_{cat}$ , and  $K_{cat}/K_m$ ) were determined using non-linear regression under initial velocity ( $v_o$ ) conditions using either C-III or C-IV as substrate.

## MATERIAL AND METHODS

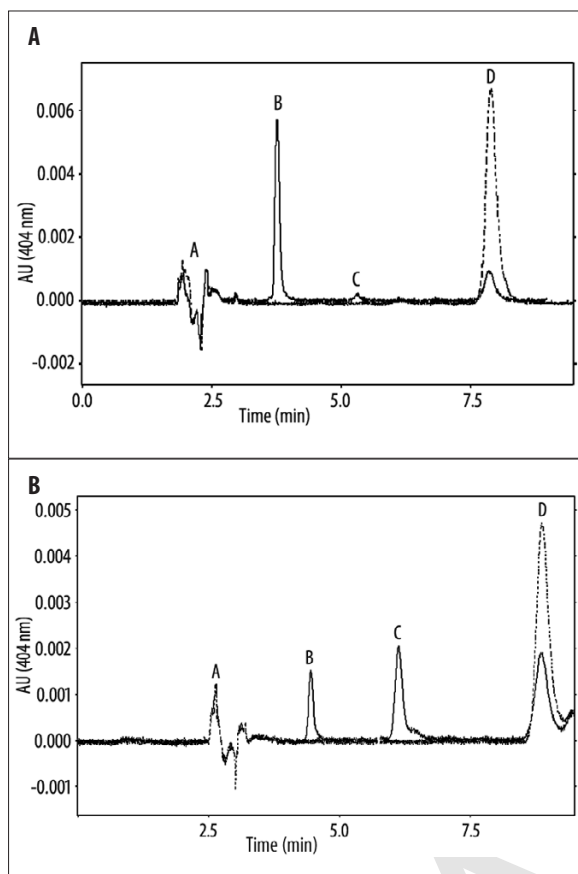
Coproporphyrin-IV tetramethyl ester was prepared by the cyclization of an a,c-biladiene intermediate following the method reported by Lash et al. [10] and coproporphyrin III tetramethyl ester was purchased from Aldrich Chemical Company. Prior to reduction to the corresponding porphyrinogens, they were incubated overnight with 8.3 M HCl to remove the methyl esters.

### Enzyme assay

Using the micro method of Jones et al. [12], either the C-III or C-IV porphyrinogen substrate (at a substrate concentration of  $1 \mu\text{M}$ ) was incubated with copro'gen oxidase at times from 15 sec to 30 min. The final reaction volume was 310  $\mu\text{l}$  which was made up of 300  $\mu\text{l}$  of enzyme in 250 mM imidazole buffer, pH 7.2 and 10  $\mu\text{l}$  of substrate. In another set of experiments, substrate concentration was varied from 0 to  $3.5 \mu\text{M}$  while holding the incubation time constant at 15 sec. For all reactions, the temperature was  $37^\circ\text{C}$  and the enzyme was  $7.5 \mu\text{g}$  (0.20 nmoles) per incubate.

The reactions were stopped by addition of 3/7 (v/v) acetic acid/ethyl acetate, followed by extraction and Fischer esterification overnight. In all experiments, a zero incubation time control (addition of acetic acid/ethyl acetate before addition of substrate) was performed for comparison. Following neutralization and extraction of the porphyrin methyl esters,



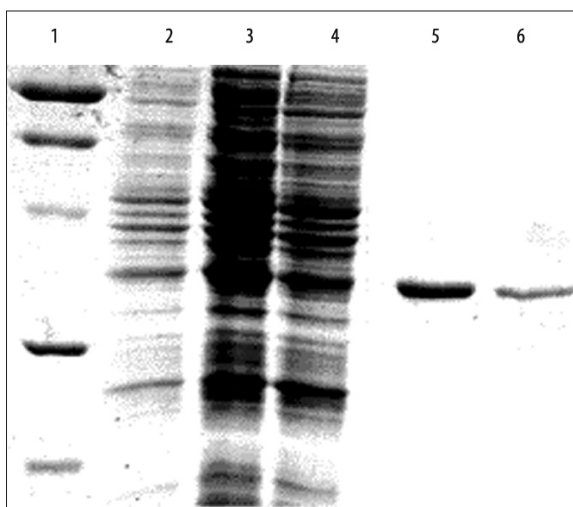


**Figure 2.** (A) Representative HPLC Chromatogram for the methyl esters of the porphyrin products resulting from incubations of C-III with pure copro'gen oxidase enzyme. (B) HPLC Chromatogram for incubations with C-IV. Zero time (unreacted) peaks shown with a dotted line and porphyrin products isolated after incubation with enzyme with a solid line. Peak A corresponds to methylene chloride (used to dissolve the sample), peak B to protoporphyrin (divinyl product), peak C to harderoporphyrin (monovinyl product), and peak D to C-III or C-IV (unreacted substrates) in Figure 2A and 2B, respectively.

analyzed using the Gold Nouveau Software and reported as % product accumulated or subsequently converted to the units of Medlock & Dailey [13]. Representative chromatograms are shown in Figure 2. Figure 2A shows product formation after zero or 20 minutes of incubation of C-III with enzyme. Figure 2B shows product formation after zero or 20 minutes of incubation of C-IV with copro'gen oxidase. In both cases the unreacted substrate, the monovinyl, and the divinyl products are well resolved.

#### Isolation and purification of copro'gen oxidase

The human cloned enzyme was grown in *E. coli* strain BL21 (DE3)RIL (Stratagene) overnight at 37°C, with shaking at 250 rpm, in one-liter cultures using a medium consisting of 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 100 mg ampicillin. Cells were isolated by centrifugation (12,000 × g, 10 min) and lysed using the French hydraulic pressure cell (20,000 psi). Then the lysate was centrifuged



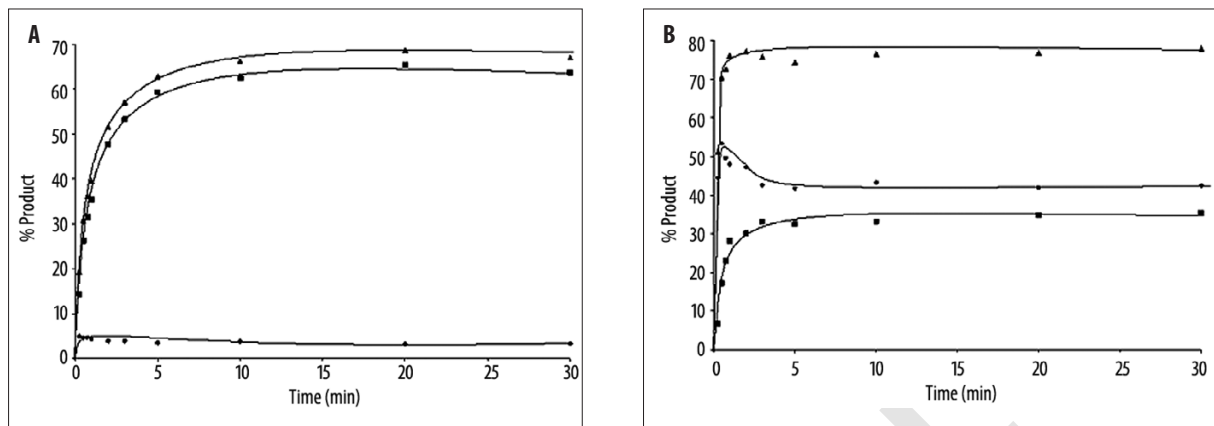
**Figure 3.** SDS-PAGE gel to show the purity of the copro'gen oxidase. In lane 1 are MW standards (97, 66, 45, 31, and 21 kDa), lanes 2 and 3 are the supernatant (1 and 10 µl), lane 4 is the Ni<sup>2+</sup> column flow through (10 µl), and lanes 5 and 6 are the elution fractions (10 and 2 µl) containing the purified enzyme.

at 35,000 × g for 30 minutes to pellet out the insoluble protein and membranes. The enzyme, which has the 6 his tag, was isolated by the Ni<sup>2+</sup> affinity procedure of Medlock and Dailey [13]. Protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli method [14] to test the purity and apparent molecular weight of the enzyme. The Bradford Protein Assay [15] was used to evaluate the concentration of the enzyme. Bovine Serum Albumin (BSA) was used as the standard.

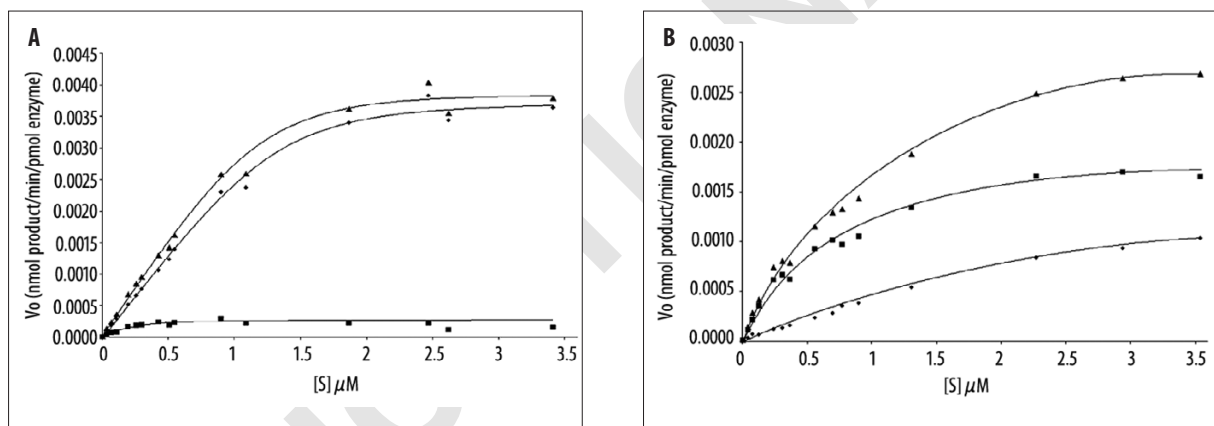
#### RESULTS

Highly purified enzyme (30 mg/liter of cell culture) was obtained and only a single band of MW 37,700 Da was observed by SDS-PAGE (Figure 3). Figure 4A shows the time dependent accumulation of divinyl, monovinyl, and total products after incubation of 7.5 µg purified enzyme with the authentic C-III substrate (1 µM). Total product was calculated as the sum of the monovinyl and divinyl products. Figure 4B shows the time dependent accumulation of divinyl, monovinyl, and total products after incubation of C-IV under the same conditions. These data are the mean of three separate experiments. The range of high and low values about the mean value was 5%. The data for the apparent linear portion of the lines ( $v_o$ ; % product/min) were evaluated by linear regression analysis. The initial velocity ( $v_o$ ) for monovinyl product accumulation is about 50 times faster ( $v_o=106$  relative to 2.4) with C-IV than with C-III. The initial velocity ( $v_o$ ) for divinyl product accumulation is about the same ( $v_o=28$  relative to 35) with either substrate. C-IV total product accumulation reached an asymptote three to four fold faster relative to C-III ( $v_o=139$  relative to 37). Using either substrate, approximately the same final total product accumulation was evident.

Figure 5A shows  $v_o$  (nmole product/min/pmole enzyme) as a function of substrate over a concentration range of 0.005 to 3.5 µM using C-III. Figure 5B shows  $v_o$  as a func-



**Figure 4.** (A) Time course study with C-III as the substrate showing the time dependency of product accumulation. (B) Time course with C-IV showing the time dependency of product accumulation. Triangles represent total product, squares show divinyl product, and circles indicate of monovinyl product intermediate accumulation. All values were the mean of three separate experiments. The range of high and low values about the mean was 5%.



**Figure 5.** Effect of substrate concentration on initial velocity ( $v_0$ ). (A) Substrate concentration curve with C-III as substrate. (B) Substrate concentration curve with C-IV as substrate. Triangles represent total product, circles show divinyl product, and squares indicate monovinyl product intermediate accumulation. The range of high and low values about the mean value of three separate experiments was 5%.

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

	Substrate	$K_m$ ( $\mu\text{M}$ )	$K_{cat}$ ( $\text{min}^{-1}$ )	$K_{cat}/K_m$
Monovinyl Product	C-III	0.13	0.20	1.50
	C-IV	0.65	1.70	2.60
Divinyl Product	C-III	0.70	3.30	4.70
	C-IV	0.83	0.70	0.85
Total Product	C-III	0.68	3.60	5.30
	C-IV	0.78	2.60	3.30

tion of substrate over a concentration range of 0.005 to 3.5  $\mu\text{M}$  using C-IV. The kinetic constants ( $K_m$ ,  $K_{cat}$ , and  $K_{cat}/K_m$ ), using either substrate, are shown in Table 1. We used the Michaelis-Menton assumptions and linear transformation of the data to obtain the kinetic constants,  $K_m$  and  $K_{cat}$ . For the production of monovinyl product there is a 5 fold increase in  $K_m$  when using C-IV as the substrate relative to C-III, but more than an 8 fold increase in  $K_{cat}$

using C-IV relative to C-III. For the divinyl product accumulated, there was essentially no difference in  $K_m$  using either substrate, but almost a 5 fold increase in  $K_{cat}$  using C-III relative to C-IV. The total product accumulation (the sum of the mono and divinyl products detected) shows about the same  $K_m$  for either substrate, and a 1.5 fold difference in  $K_{cat}$  values with C-III giving the higher turnover number.

## DISCUSSION

Using purified wildtype human copro'gen oxidase, the results clearly demonstrate that C-IV is a good substrate for this enzyme and binds in the active site with affinity comparable to the normal pathway C-III substrate. This is not surprising since C-IV can fit in either of two orientations that arise from its plane of symmetry. Our data indicate that C-IV is a better substrate for human enzyme than that of the other species tested by Buldain et al. [5]. Jackson et al. [4] reported that the monovinyl product from C-IV accumulates to about 40% at intermediary times, but was eventually converted to divinyl product. Our data show that the monovinyl product accumulates to 45% but was not apparently converted 100% into divinyl product with the human purified enzyme, even up to 30 minutes of incubation. For divinyl product accumulation, C-III results in a higher turnover (larger Kcat) and more catalytic efficiency (larger Kcat/Km) with about the same binding affinity (Km) relative to C-IV. When considering the total product accumulation, both of the substrates are equally processed under  $v_0$  conditions. From the literature, a range of Km values between 0.05 and 47  $\mu$ M for C-III have been reported for this enzyme [13]. The Km values determined here fall within the literature range. Our Km values were obtained using highly purified enzyme at a very short incubation time, while previous studies were conducted using a variety of enzyme sources, enzyme purities, incubation times, and assay procedures which may explain the vast range of values.

When the total product accumulation of C-III was compared with C-IV as a function of time, the apparent initial velocity using C-IV was about 3 fold higher than when using C-III. Thus, the position of the propionate group on the A ring leads to different kinetics for the first oxidative decarboxylation for both substrates. Different kinetics are also evident for the second oxidative decarboxylation with the accumulation of the divinyl product being much lower when using C-IV; C-IV resulted in a 5.5 fold lower Kcat/Km value relative to the C-III data.

## CONCLUSIONS

These data support the model that there is a limiting step in the second oxidative decarboxylation of C-IV. Thus our model, which predicts that C-IV comes completely out of the active site and flips over prior to re-entering the active site leading to divinyl product formation, is reasonable for the human form of the enzyme. This then would allow either C-III or C-IV to compete with the monovinyl intermediate for the active site, thus lowering the divinyl product production from either porphyrinogen. *In vivo*, this would increase the time that these porphyrinogens reside in the cell especially in the mitochondrial compartment where copro'gen oxidase is located [16], allowing them to spontaneously oxidize to the porphyrin form not utilized by copro'gen oxidase. This could, therefore, increase the severity of the porphyria. Thus detection of monovinyl porphyrins in urine need not only be related to defects in the copro'gen oxidase enzyme. Our data do not support or reject any of the proposed mechanisms for the oxidative decarboxylations performed by this enzyme [17]. The active site of the enzyme has not yet been well established although several crystal structures have been published [11]. An X-ray crystallographic structure for copro'gen oxidase has been recently solved for the *Saccharomyces cerevisiae* oxygen dependant form of the enzyme, but not the human form [11]. Although the

authors speculated about which amino acids were involved in the active site, this enzyme structure was solved in the absence of substrate. This is likely due to the difficulty of keeping the substrate in the reduced form recognized by the enzyme during crystallization. It is not clear whether the active site has yet been correctly identified. Thus, the factors influencing substrate selectivity are still not completely understood.

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