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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

## Use of Di- and Tripropionate substrate analogs to probe the active site of human recombinant coproporphyrinogen oxidase

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**Background:**

Defects in the enzyme coproporphyrinogen oxidase result in accumulation of porphyrins which may affect the severity of a subset of porphyrias. Thus evaluation of this enzyme for substrate selectivity is of value. Kinetic evaluations of recombinant human coproporphyrinogen oxidase have been undertaken using six di- and tripropionate analogs of the natural substrate coproporphyrinogen-III. These substrate analogs were modified by having alkyl groups in place of one or both of the ring 13- or 17-propionate moieties.

**Material/Methods:**

Cloned human enzyme was incubated with analogs under apparent first order conditions and with various substrate concentrations. The kinetic values,  $K_m$  and  $V_{max}$ , were determined.

**Results:**

Relative to the authentic substrate, the  $K_m$  values for the 13-ethyl, dimethyl and diethyl porphyrinogens were very comparable whereas the  $K_m$  values were much higher using dipropyl and dibutyl porphyrinogen and much lower for the 17-ethyl analog. For the dipropionate analogs, the  $V_{max}$  values were an apparent function of the carbon length of the substituent on the C and D rings, with longer carbon length severely reducing product formation by some 4–5 orders of magnitude. Also, the two isomeric tripropionates that were tested indicated that it was more detrimental to have an ethyl group at the 13-position for both binding and catalysis.

**Conclusions:**

This work extends our understanding of porphyrin ring substituent effects reported by Cooper et al. (2005). The substituents on both the C and D rings have significant effects on both the substrate binding and catalysis by this important enzyme.

**key words:**

recombinant coproporphyrinogen oxidase • substrate analogs • kinetic studies

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## BACKGROUND

The enzyme coproporphyrinogen oxidase (copro'gen oxidase, EC 1.3.3.3) is the sixth enzyme in the biosynthetic pathway for the production of heme [1]. Heme, when coupled to a globular protein, is the principal molecular oxygen carrier through out the body [1]. Malfunctions in the gene encoding copro'gen oxidase as well as other enzymes in this pathway, such as delta-aminolevulinic acid synthase, can cause a build up of porphyrins and ultimately lead to a category of diseases called porphyrias [2]. The authentic substrate for copro'gen oxidase is coproporphyrinogen-III (copro'gen-III) which undergoes two sequential oxidative decarboxylations to yield the protoporphyrinogen-IX (proto'gen-IX) product. The overall reaction catalyzed by copro'gen oxidase is shown in Figure 1. The mechanism by which copro'gen oxidase carries out the sequential oxidative decarboxylations is as yet unknown, although some studies on ring substituent effects on substrate selectivity have been reported (reviewed by Jones and Lash [3]). A model, Figure 2, of substrate binding was developed from the data obtained using substrate analogs [4]. This particular model indicates that there are a minimum of two binding sites, X and Z, as well as a catalytic site, Y. The model proposes that oxidative decarboxylations occur at the catalytic site and the substrate position is stabilized by the X and Z sites. The Z site can only accommodate a relatively small alkyl group such as a vinyl, methyl or hydrogen. The X site requires a charged group such as a propionate, and it was postulated that the substituent interacts with a positively charged amino acid. Recently, using site directed mutagenesis, arginine 262 and 401 were reported to be involved in substrate binding [5]. These binding requirements favor the sequence of peripheral substituents R Me P Me P (R=methyl, vinyl, or H, Me=methyl, P=propionate) for catalysis [4]. After the first oxidative decarboxylation on the A ring of copro'gen-III, the authentic monovinyl intermediate harderoporphyrinogen (hardero'gen) is generated. The model purposed by Lash et al. [4] suggests that a 90° clockwise turn is required to put the B ring of hardero'gen in the correct position for the second oxidative decarboxylation to generate the divinyl product, proto'gen-IX. Although it has been reported that copro'gen oxidase is a homodimer, each monomer appears to have its own active site [6,7].

To better evaluate substrate recognition of the active site of human copro'gen oxidase the use of substrate analogs that differ at the C and D ring positions have been employed. The analogs were synthesized by replacing propionates on the C and/or D ring positions with methyl, ethyl, propyl, or butyl groups [4,8]. Thus substituent effects of carbon chain length and loss of the carboxylate group could be evaluated. The porphyrinogens that were tested are shown in Figure 3. Previous studies using these di- and tripropionate substrates were done with a crude copro'gen oxidase preparation obtained from chicken red blood cells (CRBC) [4,8,9]. However, data evaluation was difficult since endogenous proto'gen-IX was present in these enzyme preparations. This endogenous product presented a challenge to quantitate the amount of newly formed product being produced from the enzyme assays. However, with the use of the recombinant human copro'gen oxidase, that particular challenge is eliminated. We now report the kinetic evaluation using highly purified recombinant enzyme using

the di- and tripropionate analogs. The kinetic constants are compared with previous data obtained using the chicken red blood cell enzyme. Using recombinant enzyme, time course studies were performed with each analog to determine the apparent first order conditions to determine the  $K_m$  and  $V_{max}$  values reported here. Also  $k_{cat}$  and  $k_{cat}/K_m$  values were calculated.

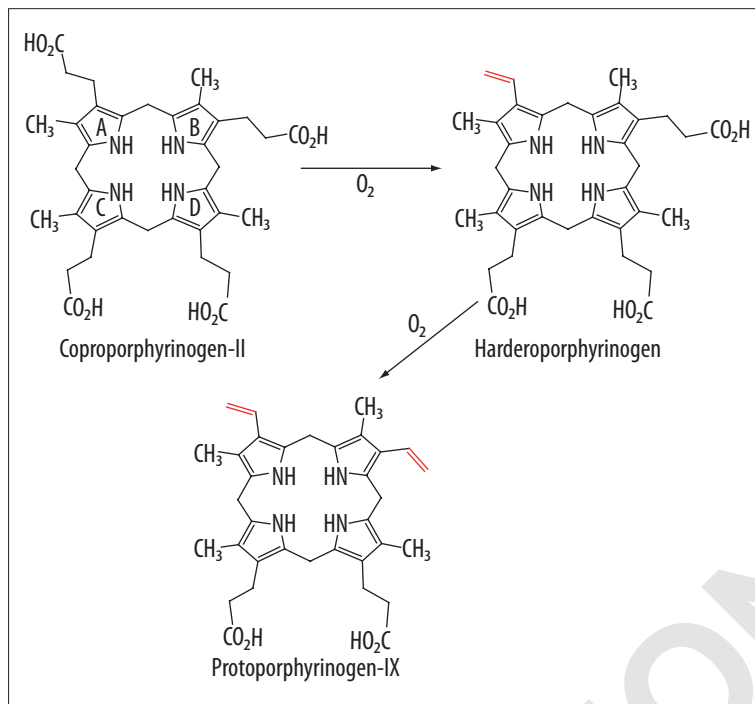
## MATERIAL AND METHODS

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

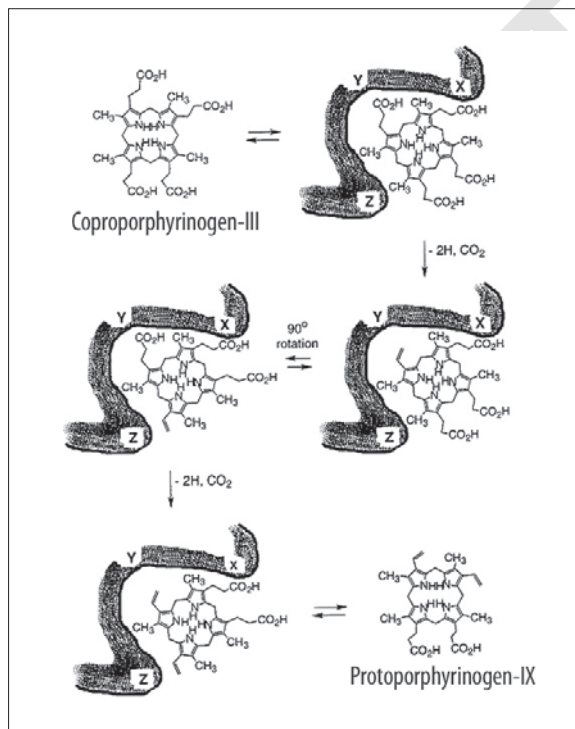
The human cloned his-tagged enzyme was expressed in *Escherichia coli* with the vector pET21d-CO containing the lac operon to better control enzyme production [5]. The *E. coli* were grown (overnight, 37°C, with shaking) in a liter of media consisting of 10 g tryptone (Fisher Chemical Company), 5 g yeast extract (Sigma Chemical Company), 10 g NaCl, 50 µg/ml ampicillin, and 34 µg/ml chloramphenicol (Sigma Chemical Company). To yield a higher concentration of protein, the cells were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) after the initial 24 hours of growth then allowed to grow for an additional 3 hours. Cells were centrifuged (10 min at 11,900 × g), and resuspended in 60 ml of resuspension buffer containing 0.0105 g of phenylmethylsulfonylfluoride (Sigma Chemical Company), 12.1 g/L Tris base, 6.9 g/L NaH<sub>2</sub>PO<sub>4</sub>, and 17.5 g/L NaCl, pH 8 then lysed using a French® hydraulic pressure cell (Sim-Aminco Spectronic Instruments) at 20,000 psi. The lysate was centrifuged for 30 min at 27,200 × g. The resulting supernatant was applied to a nickel affinity column (Qiagen), and the his-tagged enzyme was eluted with resuspension buffer containing 250 mM imidazole following the method of Medlock and Dailey [10]. The Bradford Protein Assay [11] using bovine serum albumin (BSA) as a standard was used to evaluate the concentration of the eluted protein.

### Enzyme assay

Recombinant human copro'gen oxidase was assayed using the micro method of Jones et al. [12]. In a final reaction volume of 300 µL, purified enzyme (15 µg) was incubated with 10 µL of the various freshly reduced substrate analogs (Figure 3) to give a final concentration of 0.8 µM; the dipropionate analogs [mesoporphyrinogen-VI (meso'gen-VI, diethyl porphyrinogen), dimethyl, dipropyl, and dibutyl] and tripropionate analogs [13-ethyl, 17-ethyl] were incubated at 0.5, 1, 2, 5, 10, 30, 60, or 120 minutes to determine the initial velocity ( $V_0$ ). Zero time incubations were also done as substrate stability controls. Substrate concentration was also varied under initial velocity conditions for the kinetic analysis. Reactions were carried out at 37°C, stopped with 3/7 (v/v) ethyl acetate/acetic acid, neutralized with 3% sodium acetate, extracted and Fischer esterified overnight. After esterification, porphyrins were neutralized, extracted, then separated using High Performance Liquid Chromatography (HPLC) (Beckman System Gold). A normal phase column (Beckman Silica 5 µ, 4.6 mm × 25 cm) was used with a mobile phase of 20/80 (v/v) ethyl acetate/cyclohexane at a flow rate of 1.3 ml/min; eluates were evaluated spectrophotometrically at 404 nm. Data were analyzed using the Gold Nouveau Software and reported as nmol product/min/µmol enzyme. Graphic analysis and calculated values were obtained using SigmaPlot 2000.



**Figure 1.** Overall reaction catalyzed by copro'gen oxidase of coproporphyrinogen-III to protoporphyrinogen-IX through the two sequential oxidative decarboxylations.



**Figure 2.** Active Site Model of Copro'gen Oxidase [4].

## RESULTS

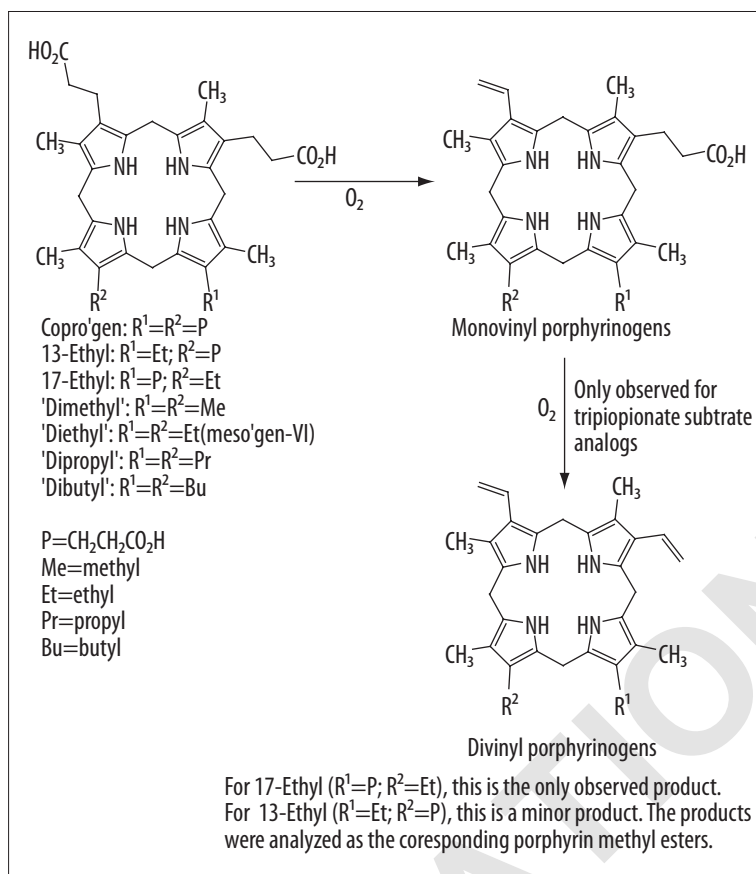
### Kinetic analysis

Shown in Figures 4–9 are the graphical representations of  $V_o$  (nmol product/min/pmol recombinant enzyme) as a function of substrate concentration ( $\mu\text{M}$ ) for all the substrate analogs tested. From these graphs  $K_m$ ,  $V_{max}$ ,  $k_{cat}$  and

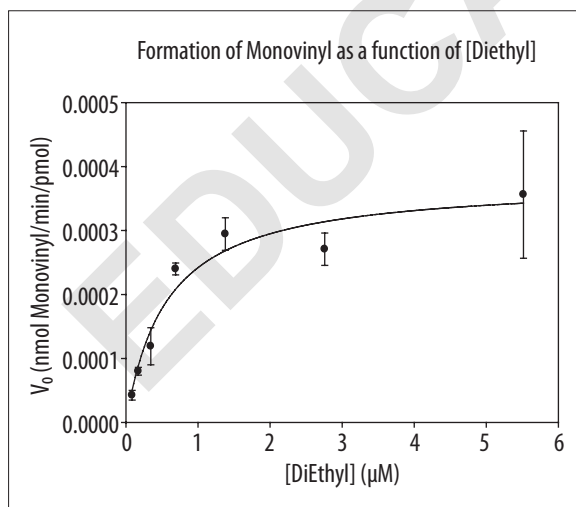
$k_{cat}/K_m$  were calculated (Table 1). Each dipropionate analog used resulted in a formation of only monovinyl product (Figure 3). Of the two tripropionates, only the 17-ethyl resulted in significant divinyl product formation whereas only the monovinyl product was generated as a major product following incubation with the 13-ethyl porphyrinogen. With the 13-ethyl analog, however, a second oxidative decarboxylation resulting in a divinyl product was noted as a minor product at longer incubation times (greater than 30 min; Figure 3). The apparent binding affinities ( $K_m$  values) for different substrates varied from 0.011 to 11.0  $\mu\text{M}$  with the 17-ethyl analog having the best affinity for the recombinant copro'gen oxidase, while the dipropyl and dibutyl analogs had the worst. Maximum velocities for each substrate analog varied widely from  $3.6 \times 10^{-7}$  to  $1.7 \times 10^{-3}$  nmol product/min/pmol enzyme with the lowest value for the dibutyl analog and the highest value for the 13-ethyl analog. The values for  $k_{cat}$ , a measurement of catalytic turnover, exhibited a range of  $3.6 \times 10^{-4}$  to  $1.7 \text{ min}^{-1}$  with dibutyl having the lowest value and 13-ethyl having the largest value. The catalytic efficiency,  $k_{cat}/K_m$ , values varied over a very wide range from  $3.4 \times 10^{-5}$  for the dibutyl analog to  $9.1 \text{ min}^{-1} \mu\text{M}^{-1}$  for the 17-ethyl analog.

## DISCUSSION

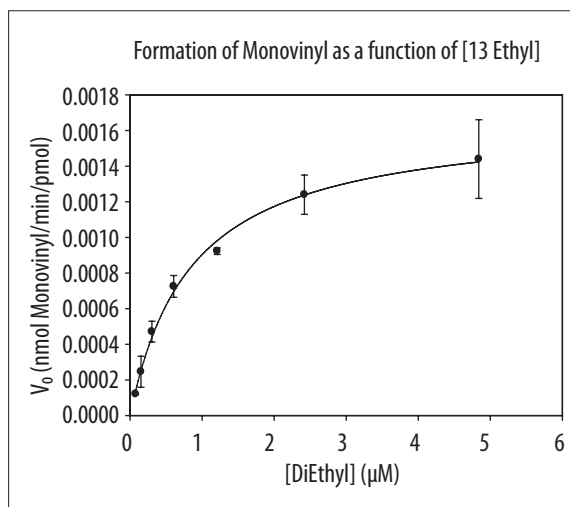
The human recombinant copro'gen oxidase recognized all of the substrate analogs studied but was only able to carry out the first oxidative decarboxylation with the dipropionate analogs. The tripropionate 17-ethyl porphyrinogen gave divinyl product with little of the intermediary monovinyl species being detected. The isomeric 13-ethyl porphyrinogen gave mostly the monovinyl product. Nevertheless, the observation that longer incubation times with the 13-ethyl analog resulted in some minor amount of divinyl product being generated is significant. When comparing all the dipropionate substrates, the dibutyl analog was the least well



**Figure 3.** Structure of Di- and Tri-propionate Porphyrinogen Analogs.



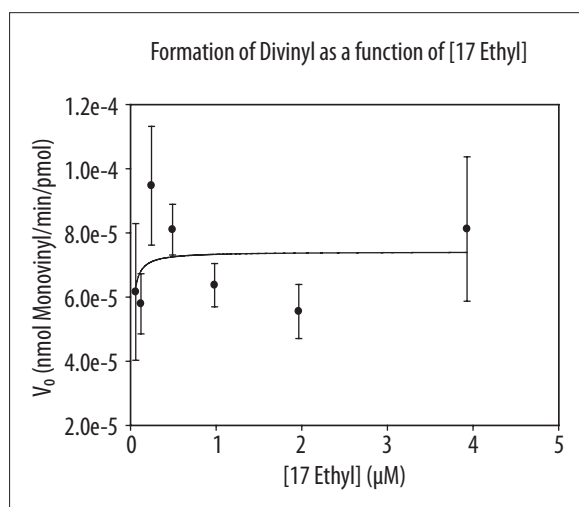
**Figure 4.** The formation of monovinyl product as a function of diethyl concentration (Incubation time – 0.5 min, mean  $\pm$  standard deviation,  $n=3$  replicates).



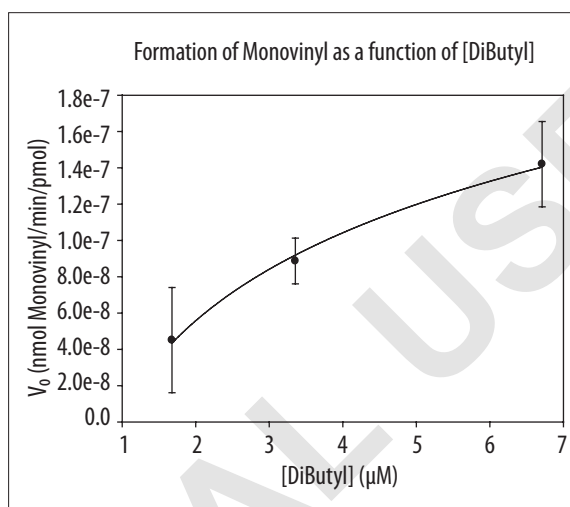
**Figure 5.** The formation of monovinyl product as a function of 13 Ethyl concentration (Incubation time – 0.5 min, mean  $\pm$  standard deviation,  $n=3$  replicates).

processed. The  $V_{max}$  for the dibutyl was almost 30 fold lower than that of the dipropyl and 3–4 orders of magnitude worse than the largest  $V_{max}$  value, 13-ethyl. Also, the  $K_m$  value was 10–12 times larger than the best analog, indicating that the binding of this dibutyl analog is severely compromised. With these bulky propyl and butyl groups present on both the C and D rings, it was predicted that the binding affinity would be affected. However, when comparing

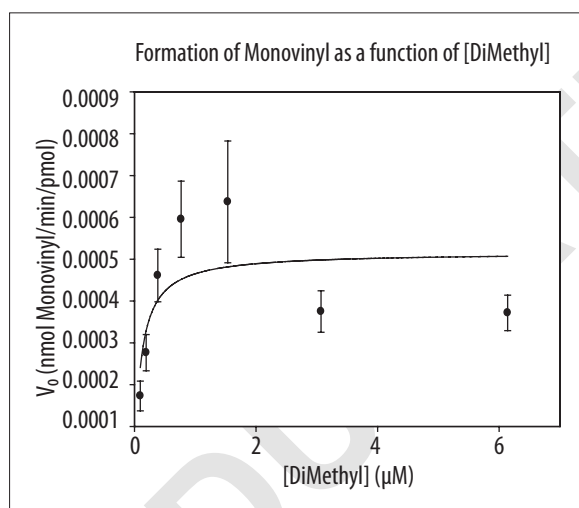
the  $K_m$  values of the dipropyl and dibutyl analogs, the values were the same implying a similar poor fit into the active site. The two analogs differ in their  $V_{max}$  values with the dipropyl having a higher  $V_{max}$  value. These data indicated that the extra methylene group on each of the C and D rings in the dibutyl slows down catalysis with no apparent difference in binding. When comparing the dipropyl and dibutyl to the kinetics obtained using diethyl (meso'gen-VI), the  $V_{max}$



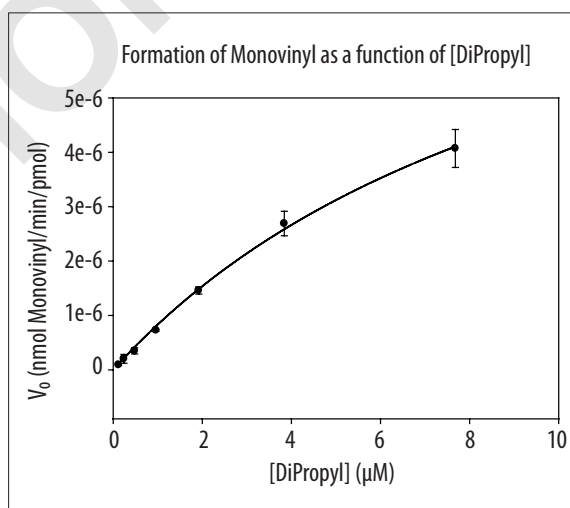
**Figure 6.** The formation of divinyl product as a function of 17 Ethyl concentration (Incubation time – 1.0 min, mean  $\pm$  standard deviation, n=3 replicates).



**Figure 7.** The formation of monovinyl product as a function of DiButyl concentration (Incubation time – 120 min, mean  $\pm$  standard deviation, n=3 replicates).



**Figure 8.** The formation of monovinyl product as a function of DiMethyl concentration (Incubation time – 0.5 min, mean  $\pm$  standard deviation, n=3 replicates).

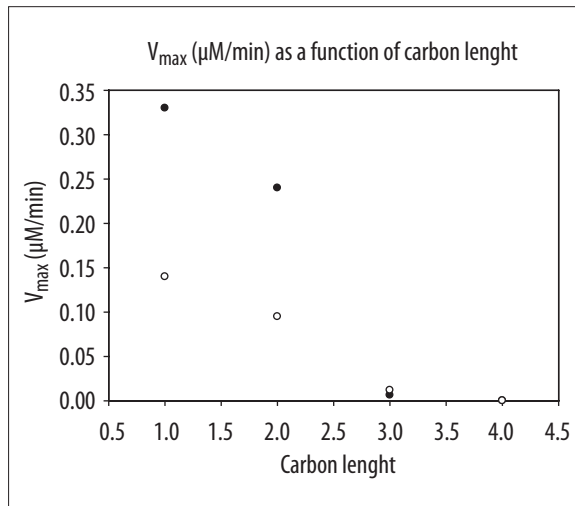


**Figure 9.** The formation of monovinyl product as a function of DiPropyl concentration (Incubation time – 120 min, mean  $\pm$  standard deviation, n=3 replicates).

for the diethyl porphyrinogen was about 3 orders of magnitude faster than that with the dipropyl and 4 orders faster than with the dibutyl analog. The  $K_m$  values for the analogs also varied by a factor of 3–4 with diethyl having a  $K_m$  value of 0.56  $\mu$ M and the dipropyl and dibutyl analogs both having values of 11  $\mu$ M. It appears that the binding affinity as well as catalysis is more favorable with shorter alkyl substituents present on both the C and D rings. This observation was further supported by the results for the dimethyl substrate analog. The  $K_m$  value for dimethyl was 0.11  $\mu$ M and the maximum velocity was found to be about the same as that of the diethyl analog. Interestingly, at higher concentrations of dimethyl this analog appeared to show inhibitory effects while the other analogs did not. With good binding affinity but moderate catalysis, the dimethyl analog at high concentrations may, in effect, occupy the active site for a longer time before it can undergo catalysis creating the apparent inhibitory effect.

The kinetic data presented here for the dipropionate analogs indicate that the shorter the carbon chain substituent present on the C and D ring the better the kinetic constants. Comparisons of kinetic constants of the crude chicken red blood cell (CRBC) enzyme preparation to the recombinant human copro'gen oxidase show a similar inhibitory trend as the substituent carbon length on the C and D rings is increased (Figure 10 and Table 2). However, the  $V_{max}$  values for the crude CRBC enzyme [9] are about half of the values for the human recombinant copro'gen oxidase.

The tripropionate analogs were synthesized with an ethyl group on either the C or D ring at the 13 or 17 positions. The 13-ethyl analog primarily underwent the first oxidative decarboxylation and a divinyl product was only formed to a minor extent. However, moving the ethyl group over to the 17-position resulted in detection of only divinyl product. Since the monovinyl product is an obligatory intermediate, we interpret



**Figure 10.** Comparison of  $V_{max}$  values for human recombinant copro'gen oxidase (●) and chicken red blood cell copro'gen oxidase (○) [9].

these data to indicate that the first oxidative decarboxylation is slower than the second reaction which supports the work of Elder et al. [13]. Thus only the second (divinyl) product is de-

tectable under our experimental conditions using the 17-ethyl analog. When comparing the  $K_m$  values of the tripropionates, 17-ethyl had a  $K_m$  value of 0.011  $\mu\text{M}$  which was surprisingly lower than the value for the authentic substrate. In contrast, the 13-ethyl had a  $K_m$  of 0.85  $\mu\text{M}$  which is very similar to that using the authentic substrate. It is clear that replacement of the propionate by an ethyl group at the 17-position affects the binding affinity more positively than the ethyl group at the position 13. However, the maximum velocity using 13-ethyl was more than an order of magnitude faster than the  $V_{max}$  using the 17-ethyl. It is not apparent why the  $V_{max}$  should be so variable between these isomeric analogs.

As had been observed in incubations with the dimethyl porphyrinogen, the use of the 17-ethyl analog showed an apparent inhibition of product formed as a function of substrate concentration. This inhibition was seen at lower concentrations (between 1–2  $\mu\text{M}$ ) than with the dimethyl (3–6  $\mu\text{M}$ ). This difference in concentration dependence could be explained by the differences in the binding affinity between each substrate. The  $K_m$  value for dimethyl was 0.11  $\mu\text{M}$  which was 10-fold higher than the 0.011  $\mu\text{M}$  value for the 17-ethyl analog. Since these  $K_m$  values are for the formation of monovinyl (using dimethyl) or divinyl product (using 17-ethyl), this may imply that the divinyl product is more able to affect interaction of enzyme with substrate.

**Table 1.** Kinetic constants for substrate analogs incubated with the human recombinant copro'gen oxidase.

Substrate	Product	$V_{max}^*$	$K_m$ ( $\mu\text{M}$ )	$k_{cat}$ ( $\text{min}^{-1}$ )	$k_{cat}/K_m$
Copro'gen-III**	Divinyl	$3.3 \times 10^{-3}$	0.70	3.3	4.7
	Total	$3.6 \times 10^{-3}$	0.68	3.6	5.3
13-Ethyl	Monovinyl	$1.7 \times 10^{-3}$	0.85	1.7	2.0
17-Ethyl	Divinyl	$1.0 \times 10^{-4}$	0.011	0.10	9.1
diMethyl	Monovinyl	$5.0 \times 10^{-4}$	0.11	0.050	0.45
diethyl	Monovinyl	$3.8 \times 10^{-4}$	0.56	0.38	0.68
diPropyl	Monovinyl	$9.9 \times 10^{-6}$	11.0	$9.9 \times 10^{-3}$	$9.1 \times 10^{-4}$
diButyl	Monovinyl	$3.6 \times 10^{-7}$	11.0	$3.6 \times 10^{-4}$	$3.4 \times 10^{-5}$

\*  $V_{max}$  – nmol product/min/pmol Enzyme; \*\* Cooper et al., 2005 [14].

**Table 2.** Comparison of human recombinant copro'gen oxidase and chicken red blood cells kinetic constants (using same units of activity) for the di- and tripropionate analogs.

Substrate	$V_{max}^*$		$K_m$ ( $\mu\text{M}$ )	
	HCPO	CRBC	HCPO	CRBC
13-Ethyl	2.6	0.21	0.85	2.0
17-Ethyl	0.048	0.055	0.011	0.88
diMethyl	0.33	0.14	0.11	1.0
diEthyl	0.24	0.095	0.56	0.99
diPropyl	0.0064	0.012	11.0	0.35
diButyl	0.00030	0.00011	11.0	0.10

\*  $V_{max}$  –  $\mu\text{M}$  Product/min; CRBC – Chicken Red Blood Cell Hemolysates [9]; HCPO – Human Recombinant Copro'gen Oxidase.



## CONCLUSIONS

It is apparent from these studies that the substituents on the C and D rings of the porphyrin are necessary for not only proper binding but also efficient catalysis. As the substituent hydrocarbon length was increased, on both the C and D rings, the enzyme kinetics were hindered relative to the authentic substrate. However, the tripropionate substrate analogs resulted in better binding affinity in the case of the 17-ethyl, and an increased rate of catalysis in the case of the 13-ethyl substitution. The formation of some divinyl product in the 13-ethyl case was unexpected and suggests that binding to site X by a propionate moiety in the active site model is not an absolute requirement for catalysis. It was interesting to note that the largest  $k_{cat}/K_m$  value (catalytic efficiency) value was calculated using the 17-ethylporphyrinogen as substrate. These data indicate that substituents on both the 13- and 17-ring positions of the substrate have important roles in binding and catalysis by the enzyme coproporphyrinogen oxidase. Thus the early model of the active site needs revision to account for these effects. As indicated in the work by Cooper et al. [14] and extended by this work, the effects of ring substituents on accumulation of porphyrin intermediates is substantial and will impact the severity of the clinical symptoms presented by porphyric patients.

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