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Kinetic Studies of Novel Di- and Tri-propionate Substrates for the Chicken Red Blood Cell Enzyme Coproporphyrinogen Oxidase[1]

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Coproporphyrinogen oxidase is an important enzyme in heme biosynthesis and catalyses the sequential oxidative decarboxylation of propionates on the A and B rings of the porphyrinogen ring. The effects of substituents on the C and D rings have not been systematically evaluated for their effects on the kinetic constants, K_m and V_{max}. A series of synthetic porphyrinogens have been tested for their ability to affect these kinetic constants for the chicken enzyme. The enzyme exhibited the largest V_{max} when incubated with the authentic substrate and was clearly able to distinguish between various substituents on the C and D rings of the macrocycle. When co-incubated with substrate, the authentic product, protoporphyrinogen-IX, appears to inhibit coproporphyrinogen oxidase and this may have an important role in the regulation of this enzyme. Thus the model for the active site of this enzyme should be modified to take these factors into account.

Key words: K_m, porphyrinogens, substrate analogs, V_{max}.

Coproporphyrinogen Oxidase (copro'gen oxidase; EC 1.3.3.3) is an enzyme found in the intermembrane space of mitochondria that plays an important role in the heme biosynthetic pathway (*1*). Defects in copro'gen oxidase or other heme biosynthetic enzymes lead to the development of a class of diseases referred to as porphyrias (*2*). The propionate groups on the A and B ring of coproporphyrinogen-III (copro'gen-III) are metabolized by copro'gen oxidase *via* an oxidative decarboxylation mechanism forming the monovinyl intermediate, harderoporphyrinogen (hardero'gen), but not isoharderoporphyrinogen, and then the divinyl product, protoporphyrinogen-IX (proto'gen-IX) (Scheme 1). Although this involves two distinct catalytic events, little work has been done to compare the rate constants for the first and second oxidative decarboxylations. Using copro'gen oxidase from a number of species, the kinetic constant, K_m, has been reported using authentic substrate on the basis of several different analytical procedures. Table I summarizes the values reported in the literature and the analytical procedures used. There is a wide range in the values reported for K_m ranging from a low of 0.05 μM for yeast grown aerobically to a high of 50 μM for *Rhodopseudomonas spheroides.*

A number of synthetic analogs have been used to probe substrate recognition at the active site of copro'gen oxidase. These studies have unambiguously shown that a specific substituent sequence (R, Me, P, Me, P where R=–CH_3, –$CH{=}CH_2$ or –H and Me=methyl, P=propionate) must be present for copro'gen oxidase to recognize and metabolize the substrate (reviewed by Ref. *3*). Catalysis begins with the first oxidative decarboxylation of the propionate moiety located at the A ring and proceeds in a clockwise manner until the required substituent sequence is no longer present (*2, 4*). From these and other data, we have developed a model (*3*) of the active site proposing one catalytic site and a minimum of two binding sites (Fig. 1) for the substituents on the 8 and 18 positions of the ring (IUPAC nomenclature).

Mesoporphyrinogen-VI (meso'gen-VI, diEt), where the propionate groups at the C and D rings are replaced with ethyl groups, has been tested as a substrate. Although this substrate is only metabolized at ring A to give a monovinyl product (Scheme 2) (*3, 18*), the kinetic data showed similar rates of meso'gen-VI metabolism when compared to copro'gen-III, suggesting that the C and D ring substituents play no major role in initial enzyme-substrate recognition. Studies evaluating the substituent sequence requirement have been extensive and insightful. However, with the exception of meso'gen-VI, few have questioned whether the substituents at the C and D rings play a role in enzyme-substrate recognition and binding as assessed by K_m and V_{max} determinations.

Our primary goal was to evaluate more thoroughly the role C and D substituents have in substrate binding and recognition. To do this we tested four synthetic dipropionate analogs where the propionate groups at both the C and D rings were replaced with methyl (diMe), ethyl (diEt), propyl (diPr), or butyl (diBu) moieties (Scheme 2) using chicken blood hemolysates (CBH) as the enzyme source. Tripropionate analogues (13Et and 17Et, Scheme 2) for which either the propionate at the 13 or 17 position was replaced with an ethyl group were also tested. For diMe, diEt, diPr, diBu, and 13Et, only the A ring propionate side chain is metabolized to give a monovinyl product and does not yield a divinyl product (*3*). However, in accord with the model (Fig. 1), 17Et did yield a divinyl product (*19*), as is the case for the natural substrate, copro'gen -III (Scheme 2). Using the time course data we have previously pub-

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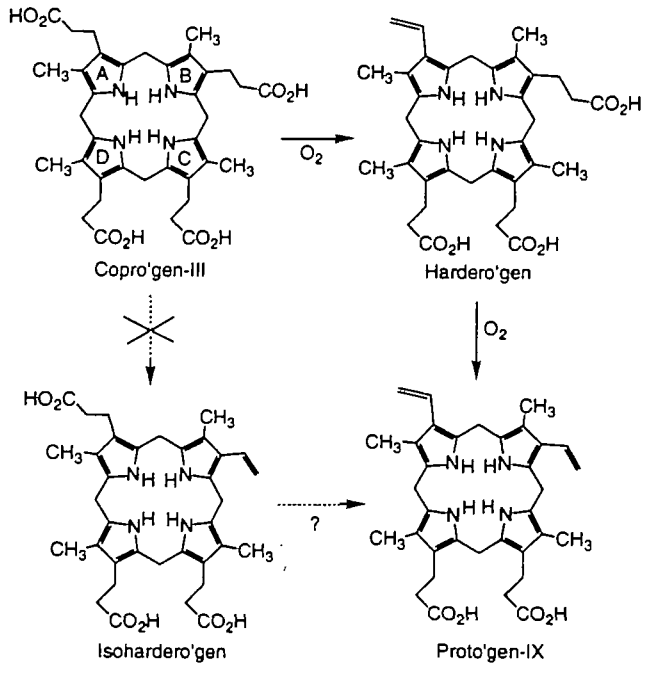


Scheme 1. **Pathway for the conversion of coproporphyrinogen-III to protoporphyrinogen-IX.**

TABLE I. **Summary of literature K_m values for copro'gen oxidase.**

Species/tissue	K_m (μM)	References
Spectrophotometric assays		
Rhodopseudomonas spheroides	30–50	*5*
Bovine liver	20–30	*6, 7*
	48	
Rat liver	30	*8*
Tobacco	36	*9*
Yeast (aerobic)	32	*10*
(anaerobic)	26	*10*
Radiochemical assay		
Human lymphocytes	0.21	*11*
Rat liver	0.16	*11, 12*
Mouse liver	0.3	*13*
Yeast (aerobic)	0.05	*14*
HPLC assay		
Human peripheral leukocyte	0.12	*15*
Fluorometric assay		
Human cloned enzyme	0.6	*16*
Human cloned enzyme-wild type	0.30	*17*
Human recombinant	0.55	*17*

lished (*3, 19*) to select appropriate first order conditions, we now report results obtained from varying the concentrations of the six synthetic analogs to allow determination of K_m and V_{max} values. An additional goal was to probe potential regulatory effects by the authentic product on the copro'gen oxidase. Although product inhibition has been previously reported by one group (*19*) using the rat liver enzyme, this has not yet been reported in other species such as chicken. In addition, several groups have noted regulation of this enzyme at the gene level by heme, O_2, and copper response elements (*21, 22*).

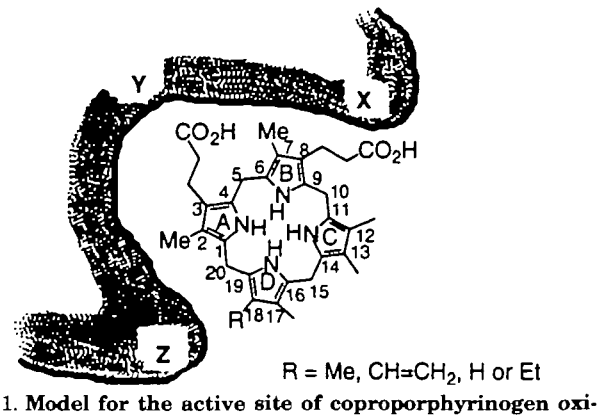
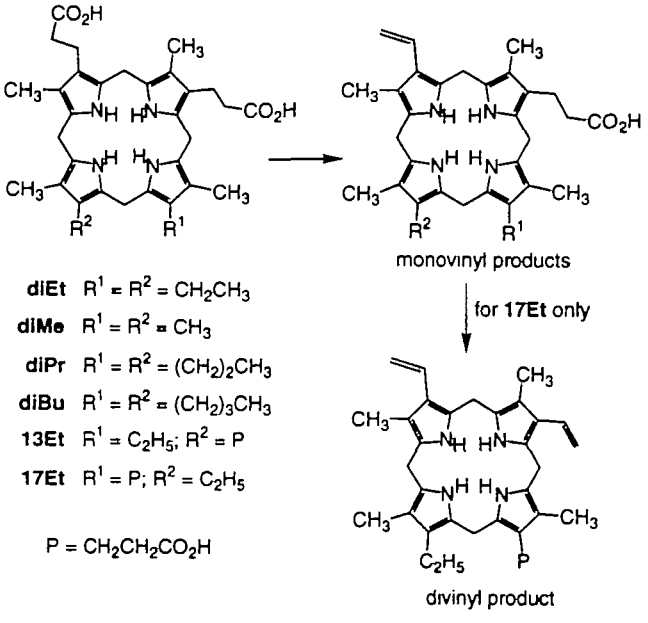


Fig. 1. **Model for the active site of coproporphyrinogen oxidase.**



Scheme 2. **Metabolism of mesoporphyrinogen-VI (diEt) and related substrate analogs.**

MATERIALS AND METHODS

With the exception of coproporphyrin III, purchased from Aldrich Chemical (Milwaukee, WI), all the porphyrins tested as substrates were synthesized as reported previously (*3, 19*). The enzyme was obtained from chicken red blood cells and prepared as reported previously (*3*). Substrates were freshly prepared from the corresponding porphyrin methyl esters. Initially, 1 ml of 25% HCl was added to the substrate (200 μg) and the mixture allowed to sit overnight in the dark. The HCl was removed *in vacuo*, and the residual porphyrin carboxylic acids were redissolved in 0.05 M KOH (200 μl) and reduced using sodium amalgam. Thioglycolate (0.1 M)/Tris-HCl (0.25 M)/EDTA (0.001 M) (pH 7.4) (900 μl) was then added to help maintain the substrate in the six hydrogen reduced state (porphyrinogen), and the solutions were then ready for immediate use.

The porphyrinogens (ranging from 0.025 to 6 μM as the final reaction concentration) were incubated with 10 ml of the enzyme source (CBH) in the dark at 37°C under initial

velocity (V_o) conditions selected from the midpoint of the apparent first order part of the product versus time curves from previous work (*3, 19*). Thus the incubation time for copro'gen-III, 13Et, diMe, and diPr was 10 min, for 17Et 20 min, for diEt 9.5 min, and for diBu 120 min. In all cases, zero incubation times (stopping solution added before substrate) were also done. Incubations were also performed with no added exogenous porphyrinogen to allow determination of the endogenous protoporphyrin-IX that is always present in our enzyme preparation. The reactions were terminated by adding 100 ml of acetic acid/ethyl acetate (3:7, v/v). The porphyrin samples were then isolated through a series of washes and extractions, re-esterified in H_2SO_4/methanol, and dissolved in 0.30 ml of dichloromethane prior to HPLC analysis. The samples were separated on a normal phase column (partisil silica, 5 μ) using different proportions of ethyl acetate and cyclohexane as the mobile phase (1:1 v/v for coproporphyrin-III; 3:7 v/v for 13Et; 2:8 v/v for 17Et, diBu, and diPr; 2.24:7.74 v/v for diMe and diEt). These solvents were used to separate the newly formed product clearly from the endogenous protoporphyrin. Porphyrin methyl esters were detected at 404 nm using a Hewlett Packard spectrophotometer. The area of each peak was determined and the percent product calculated. Initial velocity (percent product formed per min; V_o) was plotted *vs.* substrate concentration, and Lineweaver-Burk Plots were used to determine K_m and V_{max} values. In some experiments various concentrations of protoporphyrinogen-IX (from 0 to 0.06 μM) were added to the reaction tubes before the addition of 2 μM copro'gen-III substrate. The completeness of the reduction process was qualitatively assessed using a hand held UV light source since the oxidized (porphyrin) form exhibits fluorescence under these conditions and the fully reduced (porphyrinogen) form does not. These tubes were incubated for 20 min at 37°C before adding the stopping reagent. An identical set of tubes was run in parallel except that the stopping reagent was added before the addition of substrate (thus zero incubation time). The *de novo* protoporphyrinogen-IX was determined by subtraction of the zero time from the 20 min incubates. The net formation of proto'gen-IX was plotted versus the amount of proto'gen-IX added.

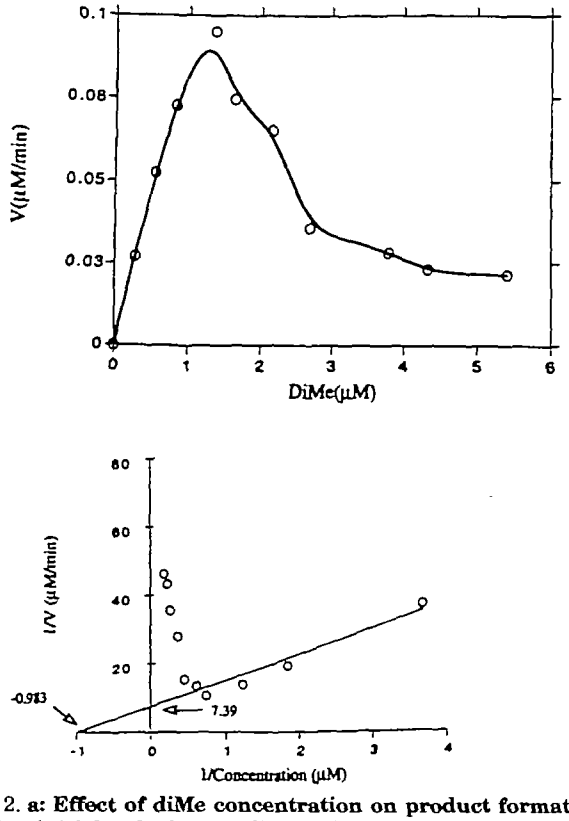


Fig. 2. a: Effect of diMe concentration on product formation under initial velocity conditions. b: Lineweaver-Burk plot for diMe.

RESULTS AND DISCUSSION

For all porphyrinogens tested, it was possible to show an apparent saturation of the enzyme with increased substrate concentrations. Figure 2a shows the effect of diMe concentration on product formation (under initial velocity conditions) and shows saturation of the enzyme at approximately 1 μM. This trend was representative of those for all seven of the porphyrinogens tested (data not shown). From this figure, it is also evident that higher concentrations of porphyrinogen did not result in an asymptote, but in an apparent decrease in the product formed under these experimental conditions. This apparent inhibition was evident for all substrates used. However, Lineweaver-Burk plots were drawn (shown in Fig. 2b for the diMe substrate) from which K_m and V_{max} values were extrapolated as shown. A comparison of the apparent binding affinity (K_m) for the various porphyrinogens as well as the apparent maximum velocity (V_{max}) is shown in Table II. The K_m values here reported are well within the range reported in the literature (Table I). It is interesting to note that the largest V_{max} value is for the authentic substrate with the second highest value for the 13Et porphyrinogen. Since the 13Et substrate can only undergo the first oxidative decarboxylation, unlike the 17Et substrate (*19*), it seems likely that non identical rate constants for the first and second oxidative decarboxylations lead to the almost 4 fold difference in V_{max} values for these two tripropionate substrates. It also is evident that the first oxidative decarboxylation is the slower step relative to the second oxidative decarboxylation, especially since the monovinyl product was not detected to any large extent in our work when either copro'gen-III or 17Et was used as substrate (*19*).

With all of the substrates tested in this study, a general trend was clearly shown that as the substrate concentration increased past a critical level, the product formed per minute (V_o) decreased. Thus either substrate or the product inhibits copro'gen oxidase. As shown in Fig. 3, the addition

TABLE II. **Calculated Michaelis-Menton values (K_m and V_{max}) for the various porphyrinogens incubated with chicken red blood cell hemosylate as an enzyme source.**

Substrate	K_m (μM)	V_{max} (μM/min)
Copro'gen-III	3.2	0.35
13Et	2.0	0.21
diMe	1.0	0.14
diEt	0.99	0.095
17Et	0.88	0.055
diPr	0.35	0.012
diBu	0.10	0.00011

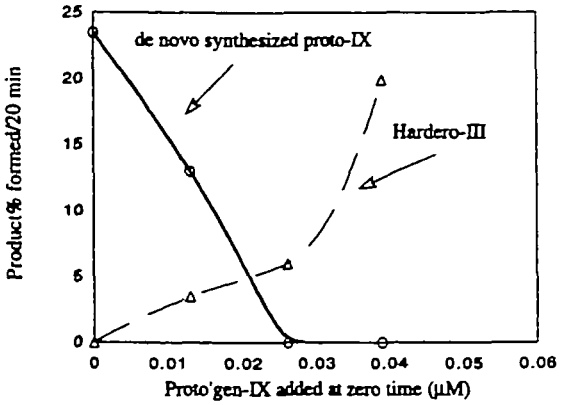


Fig. 3. **Effect of the addition of protoporphyrinogen-IX at zero time on the ability of copro'gen oxidase to process coproporphyrinogen-III.**

of authentic proto'gen-IX at zero time markedly affected the *de novo* synthesis of the divinyl product under the experimental conditions used. The newly synthesized product formed after 20 min was reduced to undetectable levels when 0.025 μM proto'gen-IX was added before the addition of copro'gen-III; however, it appears that added product does not inhibit both oxidative decarboxylations to the same extent since the monovinyl product (hardero'gen) levels increased with increasing amounts of proto'gen-IX in the incubation. Thus we see selective product inhibition of copro'gen oxidase and additional studies should be undertaken with more highly purified enzyme to determine the mechanism of inhibition.

Novel studies were conducted evaluating the role that substituents located at the C and D rings of the porphyrinogen macrocycle play in enzyme-substrate recognition. The results were compared to copro'gen-III that was also incubated with CBH. The data presented here clearly show that the substituents at these two positions play an important role in binding of the substrate to the enzyme as evidenced by the 32-fold range in K_m values between the authentic substrate and diBu, which is apparently the least well processed porphyrinogen. This is interesting since the lengths of the four carbon chain substituents are not very different from that of the propionate group. Thus the major difference involves the carboxylic acid functional group that should have a net negative charge at the pH used in these studies (pH 7.1). The differences in K_m values were not as large, however, as those seen in the V_{max} values, which differ by more than 3,000-fold between copro'gen III and the diBu. We speculate that it is the combination of both steric and ionic effects that is responsible for the observed substituent effect. The steric effects associated with the differences in carbon length and conformation of the substituents may control the "fit" of the substituent in the enzyme binding site(s), thereby affecting the rate of catalysis. The results suggest that steric interactions for neutral substituents with fewer or more than two carbons are less favorable for enzyme-substrate binding. The addition of a fourth carbon to the substituent leads to an almost complete disruption of catalysis as evidenced by the exceptionally low V_{max} value for the diBu substrate. Because the size of a butyl group is similar to that of a propionate group, the disruption observed in the metabolism of this analog is not likely to be completely the result of steric interactions. Electronic interactions between the negatively charged propionate groups and binding site amino acid(s), perhaps at the bottom of the binding pocket, may also occur, whereas a hydrophobic group, such as a butyl substituent, would not be attracted to these cationic amino acid(s) and may even experience a small repulsive force. This repulsive interaction may be strong enough to displace the substrate analog in the enzyme active site thus greatly reducing catalysis and thereby accounting for the low conversion of the analog to product observed experimentally. Thus there appears to be both a binding effect and a catalytic effect with changes in the C and D ring substituents. We need to extend our model to account for at least two additional substrate molecular recognition sites that are sensitive to C and D ring substituents.

As shown in Fig. 3, as more product was added at zero incubation time, less *de novo* product was formed, although increased amounts of the intermediate, hardero'gen, were observed with increasing concentrations of protoporphyrin-IX. It is clear that the authentic product does affect the catalysis by copro'gen oxidase and appears to affect the second oxidative decarboxylation rate more than the first since the monovinyl hardero'gen accumulates. This experiment does not, however, rule out inhibition of the enzyme by substrate. Clearly more studies are necessary using more highly purified enzyme to determine the type of inhibition exhibited by the protoporphyrin-IX. Also, the effect of product levels on the catalytic rates using Harderoporphyrinogen should be assessed. Recently, it was reported that the oxidation of protoporphyrinogen-IX in *Escherichia coli* is mediated by the aerobic coproporphyrinogen oxidase (*23*). If both porphyrinogens are substrates *in vivo* for copro'gen oxidase, this could reduce the apparent activity of the enzyme with copro'gen-III by competitive inhibition. This is interesting since all previously reported kinetic studies using CBH have contained endogenous protoporphyrin-IX, and thus the effect of its presence implies that the kinetic data using crude enzyme should be repeated using highly purified enzyme. This apparent inhibition or competition for the active site by product has important implications for the clinical sequelae for porphyria patients who often show episodic crisis events.

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