Role of aspartate 400, arginine 262, and arginine 401 in the catalytic mechanism of human coproporphyrinogen oxidase

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Abstract

Coproporphyrinogen oxidase (CPO) is the sixth enzyme in the heme biosynthetic pathway, catalyzing two sequential oxidative decarboxylations of propionate moieties on coproporphyrinogen-III forming protoporphyrinogen-IX through a monovinyl intermediate, harderoporphyrinogen. Site-directed mutagenesis studies were carried out on three invariant amino acids, aspartate 400, arginine 262, and arginine 401, to determine residue contribution to substrate binding and/or catalysis by human recombinant CPO. Kinetic analyses were performed on mutant enzymes incubated with three substrates, coproporphyrinogen-III, harderoporphyrinogen, or mesoporphyrinogen-VI, in order to determine catalytic ability to perform the first and/or second oxidative decarboxylation. When Asp400 was mutated to alanine no divinyl product was detected, but the production of a small amount of monovinyl product suggested the K_m value for coproporphyrinogen-III did not change significantly compared to the wild-type enzyme. Upon mutation of Arg262 to alanine, CPO was again a poor catalyst for the production of a divinyl product, with a catalytic efficiency <0.01% compared to wild-type, including a 15-fold higher K_m for coproporphyrinogen-III. The efficiency of divinyl product formation for mutant enzyme Arg401Ala was ~3% compared to wild-type CPO, with a threefold increase in the K_m value for coproporphyrinogen-III. These data suggest Asp400, Arg262, and Arg401 are active site amino acids critical for substrate binding and/or catalysis. Possible roles for arginine 262 and 401 include coordination of carboxylate groups of coproporphyrinogen-III, while aspartate 400 may initiate deprotonation of substrate, resulting in an oxidative decarboxylation.

Keywords: coproporphyrinogen oxidase; enzyme kinetics; catalytic aspartate

The critical roles of heme and other related metalloporphyrins are seen throughout nature as prosthetic groups crucial to the function of many proteins (Jordan 1991). In hemoglobin and myoglobin, heme is critical for their function of oxygen transport and storage, respectively. In

addition, the heme-containing cytochromes function in electron transport in the mitochondrial membrane of eukaryotic cells. The biosynthesis of heme in mammals occurs through a pathway involving eight enzyme-catalyzed reactions (Jordan 1991). The heme biosynthetic pathway starts with glycine and succinyl coenzyme A, ending with a molecule of heme containing a coordinated ferrous (Fe^{2+}) ion bound to the four pyrrolic nitrogens (Jordan et al. 1972). Coproporphyrinogen oxidase (CPO, E.C. 1.3.3.3) is the sixth enzyme in this heme biosynthetic pathway and catalyzes the conversion of coproporphyrinogen-III (copro'gen-III) to protoporphyrinogen-IX (proto'gen-IX), a divinyl product, via two sequential oxidative decarboxylations of the A and B ring propionates (Fig. 1) (Jordan et al. 1972). The conversion of copro'gen-III to proto'gen-IX

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Abbreviations: CPO, coproporphyrinogen oxidase; copro'gen-III, coproporphyrinogen-III; proto'gen-IX, protoporphyrinogen-IX; harderop'gen, harderoporphyrinogen; meso'gen-VI, mesoporphyrinogen-VI.

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Figure 1. Metabolism of porphyrinogens by CPO. Both copro'gen-III and meso'gen-VI are converted to monovinyl product. If R = P, hardero'gen is converted to divinyl product proto'gen-IX, if R = Et, a second oxidative decarboxylation is not performed.

occurs through the well-established monovinyl intermediate harderoporphyrinogen (hardero'gen), and molecular oxygen is required for the catalysis in aerobes (Kennedy et al. 1970; Jackson et al. 1980). Mesoporphyrinogen-VI (meso'gen-VI) has been shown to be a substrate for this enzyme and undergoes only the first oxidative decarboxylation (Fig. 1) (Lash et al. 1994, 1999; Jones et al. 2002). The mechanism by which CPO performs catalysis is poorly understood, in part, due to the lack of knowledge about the amino acid residues involved in active site binding and catalysis. It has been shown, however, that recombinant human CPO does not require a metal cofactor for catalysis (Medlock and Dailey 1996). CPO exists predominately as a homodimer of ~70–75 kDa with a monomeric size of ~35–37.5 kDa, and mammalian CPO is located in the mitochondria (Medlock and Dailey 1996).

The elucidation of the three-dimensional crystal structure of coproporphyrinogen oxidase from *Saccharomyces cerevisiae* (Phillips et al. 2004) and *Homo sapiens* (Lee et al. 2005) has allowed for analysis of structure–function relationships. The human and yeast forms of oxygen-dependent coproporphyrinogen oxidase each crystallized as homodimers and exhibit comparable tertiary topology. From the crystallographic data, each monomer of coproporphyrinogen oxidase contains a seven-stranded β -sheet surrounded by 10 α -helices. Upon dimerization, the β 8 moieties from each monomer interact to form a two-stranded antiparallel sheet at the middle of the dimer interface, forming key contacts for dimer stabilization. The contact surface of each monomer is comprised mainly of nonpolar amino acids, providing the foundation for a hydrophobic interface. Several conserved amino acids are located in a deep cleft, forming the putative active site of CPO, with each monomer housing its own

active site (Phillips et al. 2004; Lee et al. 2005). Using the yeast enzyme crystal structure, the substrate copro'gen-III was modeled into the cleft to locate critical amino acids (Phillips et al. 2004). The human enzyme crystallized with a molecule of citrate in the cleft, which helped probe surrounding residues in order to propose an active site (Lee et al. 2005). The cleft is formed by one side of the β -sheet and helices α 7– α 9, with α 2 located above the cleft, suggesting its role as a lid that can open and close, varying the accessibility of the active site to bulk solvent.

The conserved residues aspartate 400, arginine 401, and arginine 262 (Fig. 2) of human CPO are all thought to be contained within the proposed active site of CPO based on three-dimensional structure analysis (Fig. 3). To further the understanding of catalysis performed by coproporphyrinogen oxidase, it is important to determine if these amino acids are involved in either binding of substrate, maintenance of active site architecture, or the catalytic mechanism. Therefore, we conducted site-directed mutagenesis studies to replace each amino acid with alanine. Upon protein isolation, kinetic analyses were performed on mutated recombinant enzyme in order to determine the kinetic parameters K_m , k_{cat} and k_{cat}/K_m . Kinetic analysis of substrates with each mutated enzyme will give a more thorough understanding of the necessity of each amino acid for substrate binding or catalysis in the first, second, or both oxidative decarboxylations catalyzed by coproporphyrinogen oxidase. The ability to determine which amino acids are required for binding and catalysis will give insights into the mechanism of CPO.

Results

Wild-type recombinant human CPO and each of the purified mutant enzymes were assayed in the presence of increasing concentrations of three substrates, copro'gen-III, the natural substrate which undergoes both the first and second oxidative decarboxylations, meso'gen-VI, which undergoes only the first oxidative decarboxylation, and hardero'gen, which undergoes only the second decarboxylation. The dependence of catalytic rate on substrate concentration is illustrated in Figures 4 and 5, and the resulting kinetic parameters are summarized in Table 1.

Aspartate 400 is important for efficient catalysis

Mutation of the conserved aspartate 400 to alanine severely impaired the catalytic ability of recombinant human CPO relative to wild-type enzyme. Incubation of Asp400Ala with copro'gen-III yielded small amounts of the monovinyl product, hardero'gen, with no detectable conversion to the divinyl product proto'gen-IX. Taking into account the sensitivity of product detection (~2 nM), the amount of purified enzyme used in the assays, and the

Human	182	ER-KEGGGGISQVLTQDCVFEKAGVSI	SVVHNLSEEAQKMRSGKVLKTKD-----
Arab	114	SR-PGGGGGISRVLTQDCNVFEKAGVNSV	VVYGVMPPEAYRAAKGSASDQKP-----
Chlam	92	TR-PGGGGGISRVLTQDCNVWEKAGVNSV	VVYCTMPPEAYRAATGNAEKLKNGKD-----
Dros	120	ER-PEGGGGITCVLTQDCVFEKAGVNSV	VVTCSLPPAAVQOMRARGKNLKEG-----
Scere	45	TRGNDGGGTSMTLTQDGTTFEKGAGVNS	VVYQOLSPAASVAMKADHKNLRLPEDPKTGLP
Ecoli	37	QR-EAGGGGSRVLRNKGVFQAGVNF	SHVHCEAMPASATAHRPELAGRSF-----
Synech	54	QR-EEGGGGRSVLTADCFLEQGGVNF	SEVWCKSLPPSILKQRPPEAGHEF-----
*			
Human	232	--GKLFFCAGVSSVIHPKNPHAPTTH	ENYRYEEVEEADGNKQ---WWFGGCDLTPTTY
Arab	164	--GPVPFFAAGVSSVLHPKNPFAPTTH	ENYRYEETDAPKDVGPAPQWWFGGDTPTPAY
Chlam	146	--GRVPFFAAGISSVMHPRNPHCPTM	ENYRYEETEENWGIP---GQWWFGGDTPTPSY
Dros	171	--ASLPFFASGVSAVIHPNPHVPTTH	ENYRYEEVETAKGEKQ---WWFGGCDLTPTTY
Scere	105	VTDGVKFFACGLSMVIHPNPHAPTTH	ENYRYEETWNQDGTPTQ---WWFGGCDLTPTSY
Ecoli	87	-----EAMGVSLVHHPNHPVPTSH	ANVRFHIAEK---PGADPVWWFGGCDLTPTFY
Synech	104	-----YATGTSVVLHPKNPYIPTV	HLNRYEEAG-----PVWWFGGCDLTPTTY
Human	288	LNQEDAVHEHRTLKEACDQHGPDL	YPKFKKWCDDYEFIAHRCERRGIGGIFDDLD----
Arab	222	IFEDVKHEHHSIQKQCDKFDPSFY	PRFKKWCDDYFIKHRDERRGLGGIFDDLDN----
Chlam	201	VVPDMKHEHGTYSKAVCDRHDPAY	EKEFTWCDEYELIKHRCERRGLGGIFDDLDN----
Dros	225	LCEKASHEHQTLSACDEHDPITY	YPFKKWCDDYFRIKHRNESRGIGGIFDDID----
Scere	162	LYEEDGQLEHQLHGDALDKHDTAL	YPFKKWCDEYFYIHRKETRGIGGIFDDYD----
Ecoli	136	GFEEDAIHWHRTARDLCLPFGED	VYPRFKKWCDEYFYIKHRNEORIGGLFFDDLDN----
Synech	148	PFAEDAAHEHHTLKNACDQTHGE	FYPVKRWCEYFYIKHRCERMIGGIFDDYDQGNAP
Human	344	-----SPSKEEVFRVQSCARAV	VPSYIPLVKKHCDSSITPQOE
Arab	278	-----DYDQEMLLSFATECANS	VVPAYIPIVEKRDMEITEQEH
Chlam	257	-----DRNPEDILKFSTDAVNN	VEAYCPIIKKHMDNPTPEE
Dros	281	-----SPNQEAANFVSSCARAV	IPSIVPLVRKHKNREYGNNE
Scere	218	-----ERDPQELIKMVEDCFDA	FLPSYLTIVKRRKDMPTKEE
Ecoli	192	-----TPDFDRCFAMQAVGK	GYTDAYLPIVERKAMANGERE
Synech	208	LYRGDPDPNGPAAQYSNQLAPI	ELPWEDLFSFAQRCCGRAFLPAYSPIVEKRRNTEYGDRO
**			
Human	382	KLWQQLRRGRYVEFNLYDRGTF	GLTFPGSRIESILMSLPLTARWEYMHSESENKEAE
Arab	316	KAWQQLRRGRYVEFNLYDRGTF	GLTKTCC-RIESILVSLPLSARWEYDHKEEGTEWEK
Chlam	295	KEWQQLRRGRYVEFNLYDRGTF	GLTKTCC-RIESILMSMPQTASWLYDHQEKAGSPEAE
Dros	319	ROWQQLRRGRYVEFNLYDRGTF	GLTFPGSRIESILMSLPLHARWEYMHSEKSKSEEGK
Scere	256	QQWQQLRRGRYVEFNLYDRGTF	GLTFPGSRVESILMSLPEHASWLYNHHEAPGSPREAK
Ecoli	230	RNEQLYRRGRYVEFNLYWDRGT	FLQTCG-RTESILMSMPLVLRWEYDYQEKDGSPEAA
Synech	268	ROFQLYRRGRYVEFNLYDRGTF	GLQTCG-RTESILMSLPLVLRWQYCYSEAGSPEAE

Figure 2. Alignment of amino acid sequences of coproporphyrinogen oxidase from representative organisms. Boxed in black are identical amino acids and boxed in gray are similar amino acids. The conserved arginine 262, aspartate 400, and arginine 401 residues are marked with an asterisk. The amino acid sequence from amino acids 182–441 of human CPO (*Homo sapiens*; accession BAA04033) is compared to CPO from *Arabidopsis thaliana* (accession AAF86536), *Chlamydomonas reinhardtii* (accession AAD28475), *Drosophila melanogaster* (accession AAD46837), *Saccharomyces cerevisiae* (accession CAA89966), *Escherichia coli* (accession BAA16325), and *Synechocystis* sp. (accession BAA16863).

time of incubation (up to 2 h), the k_{cat} for divinyl product formation for Asp400Ala is <0.0005% compared to wild-type CPO. As a result, the total product accumulated is equal to that of monovinyl production. There was also no detectable product formation when Asp400Ala was incubated with either meso'gen-VI, which undergoes just the first oxidative decarboxylation, or hardero'gen, which undergoes just the second oxidative decarboxylation. When considering total product formation, the K_m value for Asp400Ala of 0.78 μM is essentially identical to the 0.79 μM value for wild-type enzyme (Table 1). The catalytic efficiency (k_{cat}/K_m) of Asp400Ala is just 0.047% of wild-type total product production, due to a 2250-fold decrease in k_{cat} . When taking into account that the molar amount of hardero'gen formed is less than the molar amount of CPO present within the assay, and that no divinyl product is being formed, Asp400Ala is

severely catalytically compromised, and is performing, on average, less than a single turnover.

Catalysis by arginine 262 and arginine 401 mutant enzymes

Mutation of arginine 262 and arginine 401 to alanine resulted in both decreased k_{cat} values and increased K_m values when compared to wild-type enzyme values using copro'gen-III as a substrate (Table 1). Replacement of Arg401 by Ala resulted in an increase in the apparent K_m value for copro'gen-III of approximately threefold and a decrease in the k_{cat} value of 16-fold for production of proto'gen-IX. Mutant enzyme Arg401Ala was able to convert meso'gen-VI to monovinyl product, although over 10-fold less efficiently than wild-type CPO, due to a fourfold higher K_m and a threefold lower k_{cat} (Table 1).

Figure 3. Three-dimensional representation of the proposed active site of human CPO. Shown is one monomer of the dimeric enzyme. The distance from the guanidyl nitrogen of Arg262 to the carboxyl carbon of Asp400 (A), the guanidyl nitrogen of Arg262 to the guanidyl carbon of Arg401 (B), and the carboxyl carbon of Asp400 to the guanidyl carbon of Arg401 (C) are shown. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen et al. 2004). Coordinates, PDB accession code 2AEX, were obtained from the Protein Data Bank (Lee et al. 2005).

Mutation of arginine 262 to alanine, however, had even more severe consequences for catalysis by CPO. The catalytic efficiency of Arg262Ala was drastically decreased, with a k_{cat}/K_m value of 0.0036%, for divinyl product formation, relative to wild-type CPO. This decrease in efficiency is attributed to a 15-fold increase in the K_m for copro'gen-III and k_{cat} decrease over 2000-fold. In contrast to Arg401Ala, no product formation was detected when mutant enzyme Arg262Ala was incubated with meso'gen-VI (Table 1). Whereas wild-type CPO is able to catalyze the conversion of hardero'gen to proto'gen-IX (J.R. Stephenson, C.L. Cooper, A-S I. M. Keck, T.D. Lash, and M.A. Jones, in prep.), we were unable to attain saturating conditions of hardero'gen with either Arg262Ala or Arg401Ala, suggesting increased K_m values in each case (data not shown). When comparing total product formation using copro'gen-III, which accounts for the sum of monovinyl and divinyl products, Arg262Ala has an 11-fold higher K_m value relative to that of wild-type CPO and a turnover number $\sim 1\%$ of the wild-type value. Arg401Ala exhibited an 11-fold increase in K_m and a k_{cat} value $\sim 44\%$ of the wild-type value (Table 2).

Discussion

Conservation of primary structure in CPO

In a multiple sequence alignment comparing CPO sequences from mammals, plants, and bacteria multiple regions of conservation are apparent, making it difficult to predict individual amino acids that are critical for catalysis. The

recently reported three-dimensional structures of CPO from yeast (Phillips et al. 2004) and humans (Lee et al. 2005) have provided information to help focus on a handful of potentially critical active site amino acids. Among these amino acids are arginine 262, aspartate 400, and arginine 401, amino acids that are conserved across species (Fig. 2). In Figure 2 as well as in a more extensive amino acid sequence alignment of ~ 20 putative oxygen-dependent CPO sequences from the protein database, arginine 262, aspartate 400, and arginine 401 are absolutely conserved.

Catalysis by uroporphyrinogen decarboxylase and porphobilinogen deaminase

Phillips et al. (2003) reported that uroporphyrinogen decarboxylase (URO-D), the enzyme that precedes CPO in the heme biosynthetic pathway, contains a catalytic aspartate residue which coordinates the four pyrrole NH groups of copro'gen-III in the three-dimensional structure. This coordination gives rise to a bowl-shaped conformation in which the pyrrolic NH groups are pointing down toward the invariant aspartate and the pyrrole rings are angled upwards. Porphobilinogen deaminase, the third enzyme in the heme biosynthetic pathway, has also been shown to contain a catalytic aspartate residue (Woodcock and Jordan 1994). Thus, the conserved aspartate 400 in CPO was selected as an important amino acid for site-directed mutagenesis studies. Likewise, the positively charged functional group of arginine makes this residue an ideal candidate for interaction with the anionic propionate moieties of copro'gen-III. It is well established that arginine residues can serve as positively charged recognition sites for anionic substrates (Riordan et al. 1977), namely the carboxylate ion moieties of copro'gen-III.

Properties of porphyrinogen molecules

It should be noted that porphyrinogens are conformationally mobile molecules. Using octamethylporphyrinogen, von Maltzan (1982) has shown that these molecules contain four nonplanar strain-free conformations, including chair, saddle, chaise longue, and the bowl forms. Although the saddle, chair, and chaise longue conformations are the most favored in solution, crystal structures of related calix[4]pyrroles show the bowl conformation was most favored in the presence of anionic binding interactions (Gale et al. 1996). This, along with the URO-D crystal structure, is consistent with a model in which aspartate 400 interacts with the pyrrolic NH groups to stabilize the bowl conformation within the active site of CPO. These data also correlate to the crystallographic data of the yeast enzyme in which the authors proposed that aspartate 274, which aligns with aspartate 400 of human CPO, was coordinating to the four pyrrolic hydrogens.

Figure 4. Initial velocity as a function of [S] plot for wild-type incubated with copro'gen-III showing kinetic parameters for total product accumulation (A), divinyl product accumulation (B), and monovinyl product after incubation with meso'gen-VI (C). Data were collected and analyzed as described in Materials and Methods. Kinetic data were analyzed by nonlinear regression analysis using SigmaPlot (Systat Software, Inc.) and are averages of triplicate determinations.

Proposed catalytic mechanism for CPO

Recently, a mechanism was proposed by Lash (2005) in which initial deprotonation of the pyrrole NH produces a species with azacyclopentadienyl anion character. The electron-rich α -carbon can then react with molecular oxygen to form a transient peroxide anion that triggers the decarboxylation of the propionate moiety. This study sought to identify active site amino acids that could be implicated in the catalytic mechanism of CPO.

Catalytic contribution of aspartate 400

Wild-type human CPO performs the second oxidative decarboxylation more readily than the first with the k_{cat} value for hardero'gen about 30 times higher than that using meso'gen-VI (J.B. Morgenthaler, R.L. Barto, T.D. Lash, and M.A. Jones, in prep.). However, the Asp400Ala mutant enzyme was unable to produce divinyl product. In addition, this mutant enzyme was an extremely poor catalyst for the first oxidative decarboxylation, producing a terminal monovinyl product at a rate $<0.04\%$ of the rate at which wild-type CPO catalyzes divinyl production. Thus, due to the large decrease in catalytic efficiency with negligible changes in the K_m for copro'gen-III, it is

highly likely aspartate 400 is a central component in the catalytic mechanism. Such a role is supported by the crystal structure of uroporphyrinogen decarboxylase (URO-D), the enzyme proceeding CPO in the heme pathway, which was cocrystallized with its product (substrate for CPO) copro'gen-III (Phillips et al. 2003). In the URO-D structure, the pyrrole units are folded upward from the plane forming a bowl shape, and thus the central pyrrolic NH groups point at the invariant aspartate 86 residue. The positive charge of the protonated pyrrole is stabilized by the negative charge of the aspartate residue, thus leading to the protonation of a pyrrole C_α atom (Phillips et al. 2003). In the case of CPO, following the mechanism proposed by Lash (2005), it can be postulated that the anionic aspartate 400 is also coordinating the pyrrolic nitrogens at the bottom of the bowl, thus leading to the deprotonation of the pyrrole NH and subsequent base-catalyzed oxidative decarboxylation of copro'gen-III.

Possible roles of arginine 262 and arginine 401 in the active site of CPO

The positive charge of an arginine side chain makes this amino acid a likely candidate for interaction with the

Figure 5. Initial velocity as a function of $[S]$ plot for mutant enzymes Asp400Ala incubated with copro'gen-III (A). Only the monovinyl product was detected; thus, monovinyl product kinetic parameters are equal to that of total product parameters for Asp400Ala. Arg262Ala incubated with copro'gen-III showing kinetic parameters for total product accumulation (B) and divinyl product accumulation (C). Arg401Ala incubated with copro'gen-III for total product accumulation (D) and divinyl product accumulation (E), or monovinyl product accumulation from incubation with meso'gen-VI (F). Data were collected and analyzed as described in Materials and Methods. Kinetic data were analyzed by nonlinear regression analysis using SigmaPlot and are averages of triplicate determinations.

negative propionate side chains of copro'gen-III in the enzyme active site. Analysis of the three-dimensional structure of the proposed active site of coproporphyrinogen oxidase (Phillips et al. 2004) implicated arginine 262 and arginine 401 as prospective binding candidates, prompting our site-directed mutagenesis studies. Upon mutation of Arg262 and Arg401, copro'gen-III K_m values

increased 15-fold and threefold, respectively. Arg401Ala retained significant catalytic function, with k_{cat} values for proto'gen-IX production at 6% and total product formation at 44% of the wild-type rate. In addition, the arginine 401 mutation did not attenuate usage of meso'gen-VI. However, not only could Arg262Ala not catalyze a divinyl product from meso'gen-VI, it had a catalytic

Table 1. Kinetic parameters of purified mutant and wild-type CPO enzymes

Enzyme	Substrate	Product	K_m (μM)	k_{cat} (min^{-1}) ($\times 10^{-3}$)	k_{cat}/K_m ($\times 10^{-3}$)
Asp400Ala	copro'gen-III	Divinyl	— ^a	—	—
Arg262Ala			5.1 ± 1.4	0.74 ± 0.07	0.15
Arg401Ala			0.96 ± 0.21	100 ± 8.0	110
Wild type			0.34 ± 0.04	1600 ± 40	4200
Asp400Ala	copro'gen-III	Total	0.78 ± 0.28	1.2 ± 0.12	1.6
Arg262Ala			8.7 ± 1.3	25 ± 1.6	2.9
Arg401Ala			2.9 ± 0.17	1200 ± 40	410
Wild type			0.79 ± 0.12	2700 ± 130	3400
Asp400Ala	meso'gen-VI	Monovinyl	—	—	—
Arg262Ala			—	—	—
Arg401Ala			2.0 ± 0.44	120 ± 10	60
Wild type			0.56 ± 0.16	380 ± 32	680

Kinetic data were obtained as described in Materials and Methods.

^aNo product was detected, with a minimum detection limit of ~ 2 nM.

efficiency for divinyl production from copro'gen-III of $<0.01\%$ of the wild-type k_{cat}/K_m value. These data suggest the side chain of Arg262 may be at a location within the active site to participate more directly in oxidative decarboxylation than Arg401. Also, due to the difference in catalytic ability to perform an oxidative decarboxylation on meso'gen-VI, it can be postulated that the two arginines are binding substrate at separate, yet fundamentally necessary, propionate moieties. The absence of propionate groups on the C and D rings of meso'gen-VI only allows the formation of a monovinyl product (Lash et al. 1994, 1999; Jones et al. 2002). The ability of Arg401Ala to perform an oxidative decarboxylation on meso'gen-VI suggests that a mutation to alanine was not detrimental to binding. Thus, arginine 401 is most likely coordinating to one or both of these C or D ring propionates, which are less important in substrate positioning in the active site, since the mutation still allowed for catalysis of the oxidative decarboxylation, although not as efficiently as wild-type CPO. However, Arg262Ala was unable to perform an oxidative decarboxylation on meso'gen-VI, indicating that arginine 262 is very impor-

tant to the binding of one or both of the propionate moieties on the A or B ring of meso'gen-VI and mutation to alanine leads to insufficient binding.

Refinement of the active site model for CPO

Through studies with porphyrinogen analogs using chicken red cell hemolysates as the enzyme source, it has been shown that modifications of B and C ring propionates reduce catalytic ability of CPO (Elder et al. 1978; Jackson et al. 1978; Yoshinaga and Sano 1980; Lash et al. 1999; Jones et al. 2002). This led to a model in which a binding site was necessary for the B ring propionate, which positioned the A ring propionate correctly for the first oxidative decarboxylation. Upon formation of monovinyl product, a binding site for the C ring propionate is necessary for the second oxidative decarboxylation to take place. Both oxidative decarboxylations are proposed to occur while the substrate is within the active site. This sequestering of the intermediate within the active site could help keep the intermediate stabilized in the reduced form to protect from oxidizing species produced during the reaction. Therefore, it can be speculated that within the dimeric enzyme, each monomer's active site performs independent catalysis of copro'gen-III to proto'gen-IX.

These studies, coupled with the kinetic parameters presented in this study, have allowed for the development of a more detailed model in which arginine 262 is involved in a binding site for the B ring propionate, and arginine 401 is the binding partner for the propionate on the C ring. The binding of copro'gen-III to these arginines puts the substrate in the correct position for the central pyrrole NH groups to coordinate with aspartate 400, allowing for the stabilization of the bowl conformation for the porphyrinogen. This interaction leads to deprotonation of the A ring NH and subsequent oxidative decarboxylation (Fig. 6).

Table 2. Ratio of monovinyl to divinyl products for purified mutant and wild-type CPO enzymes using copro'gen-III

Enzyme	Ratio monovinyl/divinyl
Asp400Ala	— ^a
Arg262Ala	32
Arg401Ala	12
Wild-type	0.50

Kinetic data were obtained as described in Materials and Methods. Hardero'gen is the monovinyl product and proto'gen-IX is the divinyl product.

^aUnable to be calculated due to lack of divinyl production.

Figure 6. Proposed CPO active site interactions of copro'gen-III with arginine 262, aspartate 400, and arginine 401. Arginine 262 forms key interactions with the propionate moiety on ring *B* and arginine 401 forms key interactions with the propionate on ring *C* of copro'gen-III. The anionic residue, aspartate 400, is proposed to play a role in catalysis by coordinating to the central pyrrolic NH groups to stabilize a bowl conformation of copro'gen-III, thus leading to initial deprotonation of the *A* ring and subsequent oxidative decarboxylation. A speculated binding partner or role for ring *D* has not yet been determined.

Effect of mutation on relative rates of the first and second oxidative decarboxylations

During catalysis utilizing copro'gen-III, a certain amount of the intermediate hardero'gen accumulates and is detected by HPLC. For the wild-type enzyme under initial velocity and saturating concentrations of copro'gen-III, the ratio of monovinyl product, hardero'gen, to divinyl product, proto'gen-IX, is ~ 0.50 (Table 2). Upon mutation of the putative active site amino acids Asp400, Arg262, and Arg401, however, the ratio of monovinyl to divinyl product changes drastically. In the case of mutant enzyme Asp400Ala, this ratio cannot be calculated since no detectable divinyl product was produced. For Arg262Ala and Arg401Ala mutant enzymes the monovinyl intermediate accumulates to a much higher concentration, with monovinyl/divinyl ratios of 32 and 12, respectively (Table 2). These data suggest that upon mutation the rate of the second oxidative decarboxylation is decreasing relative to the first oxidative decarboxylation.

CPO mutations associated with porphyria

Hereditary coproporphyria (HCP) is a genetic disorder of heme biosynthesis characterized by the excretion of coproporphyrin-III in the feces of afflicted patients

(Nordmann et al. 1983). The primary gene defect causing HCP encodes the enzyme CPO in which the activity of this enzyme is greatly reduced (Nordmann et al. 1983). Symptoms associated with HCP include photosensitivity, psychological disturbances, and abdominal pain (Elder et al. 1997). Chromosome 9 contains the gene encoding human coproporphyrinogen oxidase (Cacheux et al. 1994), and >20 different mutations in this gene causing hereditary coproporphyria have so far been identified (Martasek 1998), including mutation of two arginines (Martasek et al. 1997; Lamoril et al. 2001), one of which was investigated further in this study. It is of interest that of the three amino acids selected for mutagenesis studies, only arginine 401 has been reported as mutated in porphyric patients (Lamoril et al. 2001). An arginine 401 to tryptophan mutation in CPO was identified in a patient and was shown to possess similar functionality as a Lys304Glu mutation involved in harderoporphyria (Lamoril et al. 1995), which is characterized by a buildup of harderoporphyryn in the feces. This report is consistent with the data presented here in that the mutation of Arg401Ala resulted in accumulation of the monovinyl intermediate. We have thus proposed a role of arginine 401 in binding of substrate. The ability of the Arg401Ala mutant to perform catalysis to a greater extent than Asp400Ala and Arg262Ala supports the finding of this mutation in the population, as it is not as detrimental to overall catalysis, whereas mutation of aspartate 400 or arginine 262 may be considered highly lethal, supporting their importance in the enzyme mechanism.

Summary

The data presented here have allowed for the extension of a model for the unusual oxidative decarboxylations carried out by the essential enzyme coproporphyrinogen oxidase in the synthesis of heme. In addition, the data provide a biochemical perspective to help define a role for a documented mutation in porphyric patients. Due to the complexity of the large porphyrinogen substrates utilized by CPO, it is likely there are additional amino acids critical for binding and catalysis. Further mutagenesis studies are currently in progress on additional potential active site residues to determine their contribution to catalysis and/or binding of substrate in CPO.

Materials and methods

Construction of expression vector pET21d-CPO

Large-scale production of recombinant CPO was performed using the pET-inducible protein expression system. The NcoI/HindIII fragment from the expression vector pHHCPO (a generous gift from Dr. Harry Dailey, University of Georgia) was subcloned into the multiple cloning site of pET21d

(Novagen), resulting in expression of the cDNA encoding CPO under the control of the lac operon. Overexpression of recombinant CPO was induced with isopropyl- β -D-thiogalactopyranoside (IPTG).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the PCR-mediated overlap extension method (Horton et al. 1989) utilizing Platinum Pfx DNA Polymerase (Invitrogen). Three universal oligonucleotides and each mutagenic nucleotide (Integrated DNA Technologies) were used, resulting in recombinant human coproporphyrinogen oxidase mutants (Table 3). In each case, the conserved amino acid was changed to alanine. Oligonucleotide #1 has the sequence 5'-CGCGGATCCATGGCTCACCATCACCATCAC-3' and contained an NcoI restriction enzyme site (underlined). Oligonucleotide #2 had the sequence 5'-TTGCAATACGGAAGTTCCAGAAATTCAGCTTCTTTGG-3' and contained a 5' noncomplementary overhang of 10 nucleotides. Oligonucleotide #3 had the sequence 5'-GGGGTACCAAGCTTATTCTGCCTGCATCAACGC-3' and contained a HindIII restriction enzyme site (underlined). The encoded wild-type CPO lacked the mitochondrial-targeting signal located on 100 amino acids at the amino terminus. The sequence Met-Ala-His-His-His-His-His was encoded at the amino terminus prior to proline 34 to serve as a 6x-His-Tag for affinity purification using a metal affinity column. Restriction endonucleases NcoI and HindIII were used to digest each PCR product, which were then ligated with NcoI and HindIII digested pET21d. The cDNA of each mutant was sequenced to verify the mutation using automated fluorescent sequencing on a 3130 Genetic Analyzer using a BigDye terminator DNA sequencing kit from Applied Biosystems.

Expression and purification of His-tagged wild-type and mutant CPO proteins

Escherichia coli strain BL21(DE3)RIL (Stratagene) was used as the expression host. One liter cultures containing pET21d-

CPO were grown in a shaking incubator at 37°C and 300 rpm to an optical density of 0.8–1.0 at 600 nm in Luria Broth medium with antibiotics (ampicillin and chloramphenicol at 100 μ g/mL and 34 μ g/mL, respectively). Overexpression of protein was induced by the addition of 1 mM IPTG followed by incubation at 37°C with 300 rpm shaking for ~3 h. *E. coli* cells expressing either wild-type or mutant forms of CPO were harvested by centrifugation at 4620g for 5 min. Bacterial cell pellets were resuspended in 40 mL of CPO resuspension buffer (50 mM NaH₂PO₄, 300 mM NaCl, 50 mM Tris at pH 8) and lysed using a French Pressure Cell at 20,000 psi with the addition of phenylmethylsulfonylfluoride as a protease inhibitor. The lysed cells were centrifuged at 27,200g for 30 min and the supernatant was passed through a 4-mL column of Ni-NTA agarose metal affinity resin (Qiagen). The column was washed with resuspension buffer containing 20 mM imidazole and desired protein was eluted with resuspension buffer containing 250 mM imidazole at a final pH of 7.0. The protein concentration of each fraction was determined by the method of Bradford (1976) using a Bio-Rad Protein Assay kit and BSA as a standard, and purification was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 7).

Substrate preparation

Three different substrates were used for kinetic analysis of recombinant enzymes. The use of these three substrates allowed for the relative comparison between the ability of wild-type and mutant enzymes to perform the first and second oxidative decarboxylations. Each substrate was prepared from the corresponding porphyrin methyl esters by incubation overnight with 8.3 M HCl and reduction with 3% sodium amalgam as previously reported (Lash et al. 1999; Jones et al. 2002). Coproporphyrin-III tetramethyl ester was purchased from Aldrich Chemical. Harderporphyrin trimethyl ester and mesoporphyrin-VI dimethyl ester were previously synthesized (Lash et al. 1999, 2001, 2002). The reduced porphyrinogens were used directly after reduction and concentrations were quantitated spectrophotometrically at 406 nm in 8.3 M HCl.

Enzyme assay

Recombinant wild-type and mutant CPO were assayed at 37°C using the microprocedure previously reported (Jones et al. 2003). Following incubation and extraction, the porphyrins were converted to their respective methyl esters and separated by HPLC using a normal phase silica column and a mobile phase of ethyl acetate:cyclohexane (35:65 [v/v] for coproporphyrin-III and harderporphyrin and 25:75 [v/v] for mesoporphyrin-VI). Porphyrins were detected at 404 nm, and amount of product was calculated from the integrated peak areas. Initial velocity (V_0) was evaluated for each mutant with each substrate using incubation times ranging from 15 sec to 20 min. For kinetic analysis, both wild-type and mutant enzymes were assayed under apparent initial velocity conditions with the three substrates at various concentrations from 0.05 to 25 μ M. Kinetic data were performed in triplicate and analyzed by nonlinear regression analysis using SigmaPlot. Data are reported for formation of monovinyl intermediate, divinyl product, and the sum of these (indicated as total product) for both wild-type and mutant enzymes and kinetic parameters are given in Table 1.

Table 3. Wild-type CPO cDNA sequence aligned with mutagenic oligonucleotides for Arg262, Asp400, and Arg401

Arg262Ala	5'-CATTTCAACTACGCGTACTTTGAAGTAGAAG-3'
Wild type	5'-CATTTCAACTACAGATACTTTGAAGTAGAAG-3'
Asp400Ala	5'-GAATTTAATCTGCTGTATGCCCGGGGCACA AAGTTTGGC-3'
Wild type	5'-GAATTTAATCTGCTGTATGATCGGGGCACA AAGTTTGGC-3'
Arg401Ala	5'-GAATTTAATCTGCTGTATGATGCCGGGCACA AAGTTTGGC-3'
Wild type	5'-GAATTTAATCTGCTGTATGATCGGGGCACA AAGTTTGGC-3'

Shown are the sequences of wild-type cDNA sequence aligned with the codons for Arg262, Asp400, and Arg401 and the oligonucleotides used for PCR mutagenesis. Underlined are the codons corresponding to Arg262, Asp400, and Arg401 of wild-type CPO as well as the codon change for each mutant. Highlighted are the restriction enzyme sites added as silent mutations. Arg262Ala contained a SmaI restriction enzyme site, Asp400Ala contained a MluI restriction enzyme site, and Arg401Ala contained a NaeI restriction enzyme site.

Figure 7. SDS-PAGE (12% polyacrylamide) of purified wild-type CPO, Arg262Ala, Asp400Ala, and Arg401Ala. Approximately 2–3 μ g of protein were added per lane.

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