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B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The catalytic contribution of four conserved histidines of human coproporphyrinogen oxidase (CPO) has been investigated using site-directed mutagenesis to change histidine (H) into alanine (A).

Material/Methods: The wild-type and mutant enzyme forms were analyzed for their ability to utilize coproporphyrinogen-III, mesoporphyrinogen-VI, and harderoporphyrinogen as substrates.

Results: Wild-type CPO had specific activities of 4.9±0.9 nmole product/min/mg for coproporphyrinogen-III, 1.7±0.7 nmole product/min/mg for mesoporphyrinogen-VI, and 5.1±1.8 nmole product/min/mg for harderoporphyrinogen. The four mutant enzymes were catalytically competent with all three substrates, but to varying degrees. The most affected mutant was the H158A enzyme which exhibited approximately 50-fold lower activity than wild-type recombinant CPO.

Conclusions: Thus, His158 of human CPO may have a role in the active site, but none of the conserved histidine residues of human coproporphyrinogen oxidase is essential for catalytic activity although changes in histidines have been implicated in the disease state hereditary coproporphyria.

key words: **catalysis • heme • histidine • mutagenesis • porphyrin**

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BACKGROUND

Heme and related porphyrins serve as important prosthetic groups in a number of biologically critical proteins, including hemoglobin, essential for oxygen transport in the blood, and the cytochromes, important in electron transport in the mitochondria of eukaryotic cells. Heme biosynthesis is accomplished through a series of eight enzyme-catalyzed reactions. The Shemin pathway (of plants and animals) starts with the relatively simple molecules glycine and succinyl coenzyme A, and ends with a heme molecule containing four pyrrole rings linked by methine bridges capable of coordinating a ferrous (Fe^{2+}) ion. Coproporphyrinogen oxidase (CPO; EC 1.3.3.3) is the sixth enzyme of the heme biosynthetic pathway, catalyzing the sequential oxidative decarboxylation (Figure 1A) of two propionate groups of coproporphyrinogen-III to vinyl groups to yield protoporphyrinogen-IX [1,2]. This is a two-step process for which the monovinyl intermediate (harderoporphyrinogen) has been well established. The enzyme from aerobic organisms requires molecular oxygen, but no other known cofactor is required. Medlock and Dailey [3] specifically and convincingly report that the recombinant human enzyme expressed in *Escherichia coli* is not a metalloprotein. In addition to the authentic substrate, synthetic analogues have also been evaluated as substrates for CPO. Mesoporphyrinogen-VI (Figure 1B) has been shown to be a substrate for this enzyme but only undergoes the first oxidative decarboxylation [4,5].

Coproporphyrinogen oxidase deficiency in humans leads to the disease state hereditary coproporphyria (HCP). In HCP, CPO activity is often significantly reduced, and afflicted HCP individuals suffer from neurological disturbances and cutaneous photosensitivity [6,7]. The gene encoding human CPO is located on chromosome 9 [8] and genome sequencing efforts have revealed mutations in the CPO gene that lead to HCP [9,10]. In mammals, CPO is expressed as a 40 kDa precursor, and contains an amino terminal mitochondrial targeting signal. After proteolytic processing, the protein is present as a mature 37 kDa form [10–12]. CPO is reported to be located in the intermembrane space of the mitochondria and possesses the ability to associate with the mitochondrial inner membrane [1,13].

Coproporphyrinogen oxidase from both prokaryote and eukaryote organisms has been studied, including yeast [14,15], chicken erythrocytes [16], mouse liver [17], and bovine liver [18]. In addition to the isolation of CPO from bovine liver, mouse liver, and yeast, recombinant human [3] and mouse CPO [19] have been purified. Studies investigating the chemical modification of specific amino acids have implicated tyrosine, arginine, histidine and lysine residues as playing a significant role in catalysis or protein structure [16,20,21].

Modern molecular biological techniques and genome sequencing projects have facilitated the influx of a tremendous amount of nucleotide sequence data. A search of current nucleotide and protein databases reveals more than 20 putative CPO sequences, from organisms as diverse as mammals, plants, insects, yeast, enterobacteria, and cyanobacteria. An alignment of the available CPO amino acid sequences is useful for assessing the common evolutionary origin of the enzymes in this family. Certain amino acids have been conserved in evolution, suggesting a critical role in the function of the protein. Among the conserved amino acids of aerobic CPO are four histidines, an amino acid previously implicated as potentially critical for catalysis by chemical modification studies [21]. Shown in Figure 2 is an alignment of CPO amino acid sequences from seven representative organisms. Among these seven sequences, the overall amino acid sequence identity is 26%. Identical amino acids are shaded in black while the four conserved histidines at positions 148, 158, 197, and 227 are indicated by the asterisks. The human and the mouse genes are highly conserved with both having the four conserved histidines as compared by Medlock and Dailey [3].

Catalysis by CPO is poorly understood at the molecular level. The roles of specific amino acids in catalysis by human CPO have not yet been elucidated. Histidines are common proton donors and acceptors in biological catalysis, and therefore their involvement in the catalytic mechanism of CPO is reasonable to assess. Conserved His158 was previously proposed to play a critical role in metal coordination in mouse CPO [19]. Subsequently, however, human CPO was found to not require metal ions for catalysis [3].

This study investigated the contribution of four conserved histidines to catalysis by human coproporphyrinogen oxidase by substituting histidine (H) at position 148, 158, 197, or 227 with alanine (A) using site-directed mutagenesis. The wild-type and mutant forms of the recombinant enzyme were expressed in *E. coli*, purified to electrophoretic homogeneity, and analyzed for the ability to utilize coproporphyrinogen-III, harderoporphyrinogen, and mesoporphyrinogen-VI as substrates for catalysis. Use of the three different substrates allows for comparison of rates for the first and second decarboxylations. For the most interesting histidine mutant (H158A), enzyme kinetics were also carried out to determine K_m, V_{max}, k_{cat} and k_{cat}/K_m, and these were compared to the values obtained for the recombinant human wild-type enzyme.

MATERIAL AND METHODS

Material

The polymerase chain reaction was performed using oligonucleotide primers and Platinum *Pfx* DNA Polymerase from Invitrogen. Expression vector pET21d was obtained from Novagen. Restriction endonucleases were purchased from New England Biolabs. T4 DNA Ligase was from Promega and isopropyl-β-D-thiogalactoside (IPTG) was from Eppendorf. Clontech was the source of TALON metal affinity resin. Protease inhibitors were from Sigma. Qiaprep spin miniprep and Qiaex II gel extraction kits were from Qiagen. Automated fluorescent sequencing was performed on an ABI 377 sequencer using a BigDye terminator DNA sequencing kit from Applied Biosystems. Tryptone, yeast extract, sodium chloride, chloramphenicol, and ampicillin were purchased from Fisher. The Protein Assay Kit was from Bio-Rad.

Construction of expression vector pET21d-CPO

The pET inducible protein expression system was used for large-scale production of recombinant CPO.

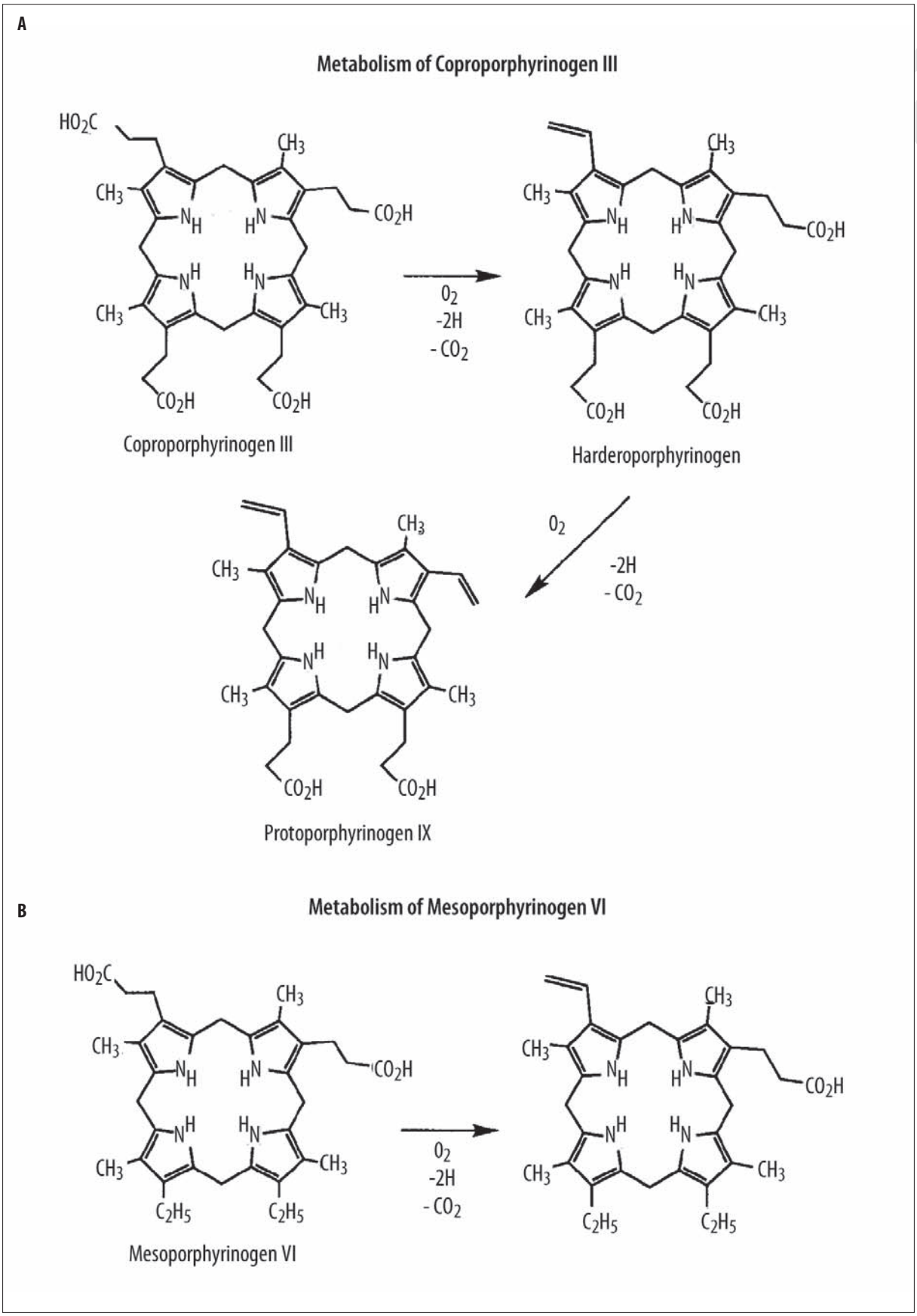


Figure 1. Reactions catalyzed by coproporphyrinogen oxidase. (**A**) conversion of coproporphyrinogen-III to protoporphyrinogen-IX via harderoporphyrinogen, a monovinyl intermediate. (**B**) conversion of mesoporphyrinogen-VI to the monovinyl product.

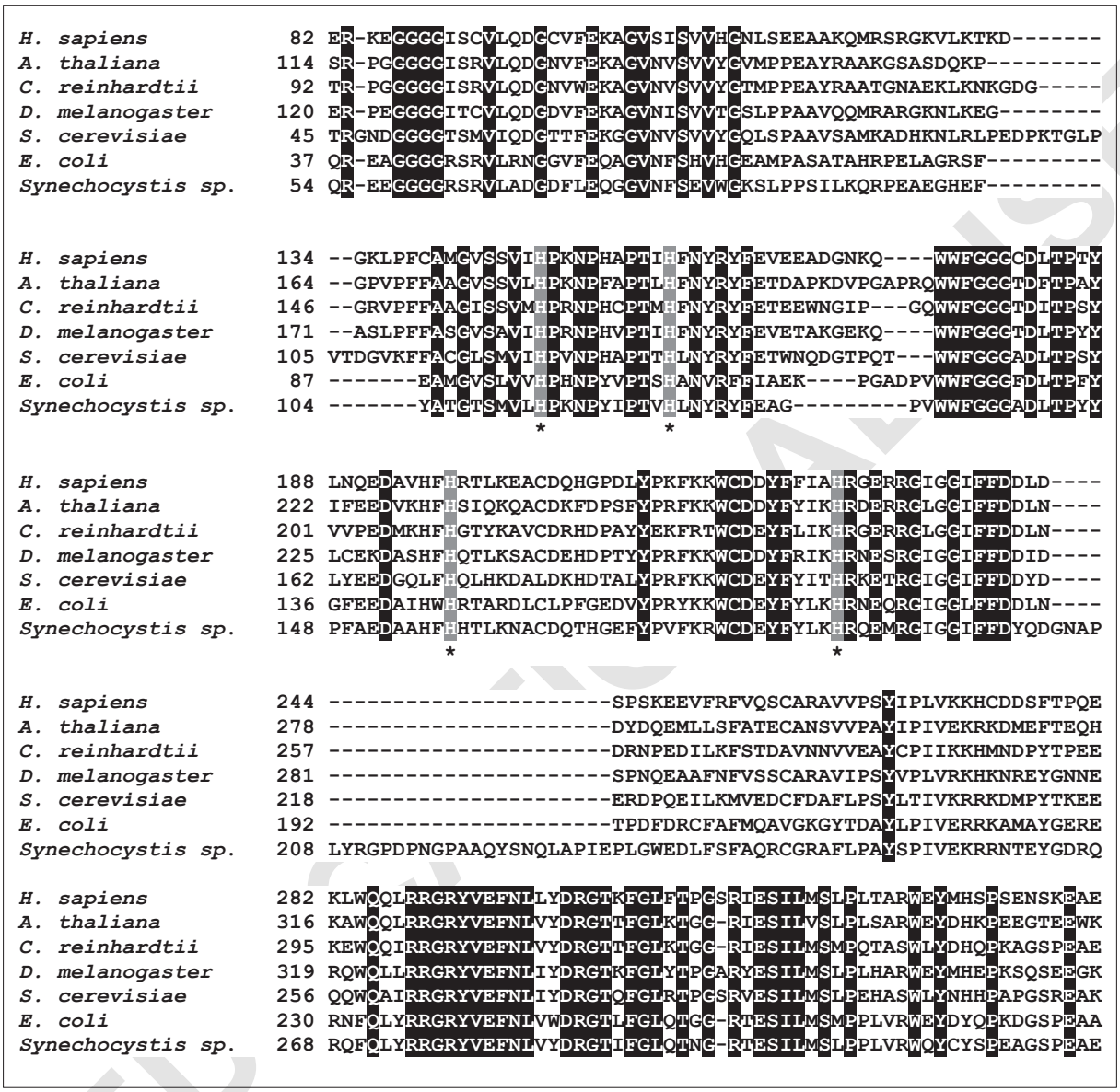


Figure 2. Alignment of amino acid sequences of coproporphyrinogen oxidase from representative organisms. The amino acid sequence from amino acids 82-341 of human coproporphyrinogen oxidase (*Homo sapiens*; accession BAA04033) is compared to coproporphyrinogen oxidase from a plant (*Arabidopsis thaliana*; accession AAF86536), a green algae (*Chlamydomonas reinhardtii;* accession AAD28475), fruit fly (*Drosophila melanogaster*; accession AAD46837), baker's yeast (*Saccharomyces cerevisiae;* accession CAA89966), an enterobacteria (*Escherichia coli*; accession BAA16325), and a cyanobacteria (*Synechocystis sp.*; accession BAA16863). Conserved amino acids are boxed in black. The conserved histidines at positions 148, 158, 197, and 227 are boxed in gray and indicated by an asterisk. Sequence alignment was generated using the BCM Search Launcher at Baylor College of Medicine (*http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html*) and the Boxshade server (*http://www.ch.embnet.org/software/BOX_form.html*).

The NcoI/HindIII fragment from expression vector pHHCPO [3] was subcloned into the multiple cloning site of pET21d. This resulted in expression of the cDNA encoding CPO to be under control of the lac operon. Production of recombinant CPO is induced by the addition of IPTG.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the PCR-mediated overlap extension method [22] utilizing three general oligonucleotides and a fourth mutagenic oligonucleotide. Oligonucleotide #1 had the sequence 5'-CGCGGATCCAT GGCTCACCATCACCATCACC-3' and contained a NcoI restriction enzyme site (underlined). Oligonucleotide #2 had the sequence 5'-TTGCAATACGGAACTTCCAGAATTTCA GCTTCTTTGG-3' and contained a 5' non-complementary overhang of 10 nucleotides. Oligonucleotide #3 had the sequence 5'-GGGGTACCAAGCTTATTCTGCCTGCATCA ACGC-3' and contained a HindIII restriction enzyme site (underlined). Mutagenic oligonucleotides had the following sequences (alanine codons underlined): H148A; 5'-GG GCGTGAGTTCTGTTATCGCCCCCAAGAATCCTCATG-3', H158A; 5'-CCTCATGCTCCTACGATCGCGTTCAACTACA GATAC-3', H197A; 5'-GAAGACGCTGTCCATTTTGCGCG

Table 1. Kinetic constants of coproporphyrinogen oxidase for total product accumulated.

	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m
Wildtype Enzyme	Coproporphyrinogen-III	0.3	3.6	12.0
	Harderoporphyrinogen	1.2	7.9	6.6
H158A Mutant Enzyme	Coproporphyrinogen-III	0.15	0.015	0.10
	Harderoporphyrinogen	0.15	0.023	0.15

CACTCTGAAGGAGGC-3', H227A; 5'-GATTACTTCTTTAT AGCCGCCCGTGGAGAGCGGCGGGGC-3'.

PCR was run using the following thermocycle parameters: 94°C 30 seconds, 55°C 30 seconds, 72°C 1 minute. Thirty cycles were run in each PCR reaction. Three PCR reactions were run for construction of each mutated cDNA. PCR reaction 1 employed oligonucleotides #1 and #2. Oligonucleotide #1 anneals to the bottom strand (non-coding strand) at the 5' end of the cDNA. Oligonucleotide #2 anneals to the top strand (coding strand) near the 3' end of the gene and contains 10 mismatched nucleotides at the 5' end. PCR reaction 1 resulted in a DNA fragment that has a mismatch at one end encoded in oligo 2, preventing extension from its 3' end in subsequent PCR reactions, providing the basis for selection of mutant CPO clones. PCR reaction 2 used oligonucleotide #3 and the desired mutagenic oligonucleotide, and resulted in a DNA fragment complementary to the PCR reaction 1 fragment. Template DNA for PCR 1 and PCR 2 was pET21d-CPO, the wild-type human CPO cDNA cloned into pET21d. The encoded wild-type CPO lacked the amino terminal 33 amino acids that serve as a mitochondrial targeting signal, and contained the amino terminal amino acid sequence Met-Ala-His-His-His-His-His-His preceding proline 34, serving as a 6x-His-tag for affinity purification using a metal affinity column. Following PCR 1 and 2, 10 μl of each PCR reaction was run on a 0.7% agarose gel. The desired DNA fragments were purified from the gel and combined in a third PCR reaction employing oligonucleotides #1 and #3. Each PCR product was digested with restriction endonucleases NcoI and HindIII, followed by ligation with NcoI/HindIII-digested pET21d. Each cDNA was sequenced to verify the desired mutation had been incorporated.

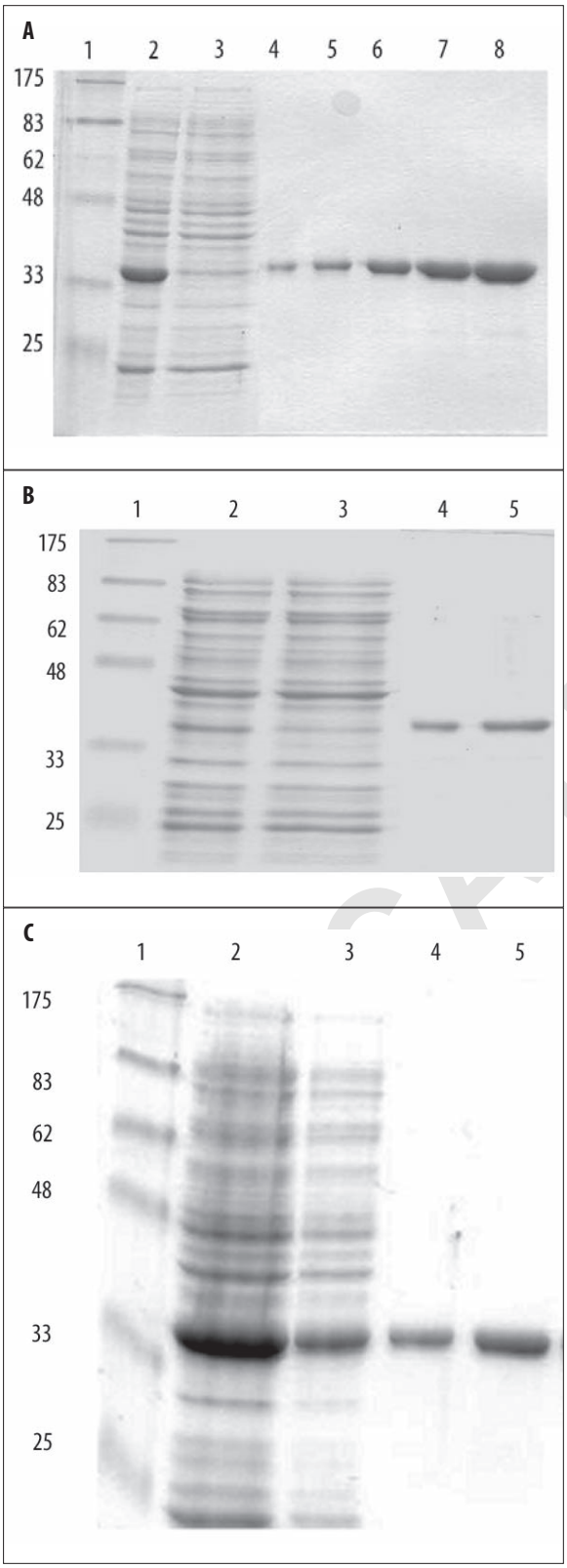
Expression and purification of His-tagged wild-type and mutant CPO proteins

Escherichia coli strain BL21(DE3)RIL (Stratagene) was used as 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 at 600 nm in LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. Induction of protein expression was accomplished by the addition of 1 mM IPTG, followed by incubation at 37°C and 300 rpm shaking for 3 hours. Cells expressing wild-type and mutant forms of CPO were harvested by centrifugation at 4,620 × g for 5 minutes. A considerable percentage of mutant enzyme H197A was present in inclusion bodies when expression was performed at 37°C. Therefore, upon induction with IPTG, bacterial cultures expressing H197A were grown at 25°C, significantly increasing the amount of soluble protein.

Bacterial cell pellets were resuspended in 40 ml CPO lysis buffer (50 mM Na_xPO_4, 300 mM NaCl, 50 mM Tris, pH 7) containing 2.5 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, 1 μg/ml antipain, 10 μg/ml p-aminobenzamidine, 10 μg/ml benzamidine, and 0.2 mM phenylmethylsulfonylfluoride. Cells were lysed using a French Pressure cell at 20,000 psi, therefore addition of the detergent n-octyl-β-D-glucopyranoside, used previously by Medlock and Dailey [3], was unnecessary. We observed similar enzymatic activity for CPO in the presence or absence of detergent (data not shown). After centrifugation (100,000 × g for 30 min) the supernatant was passed through a 4 ml column of TALON metal affinity resin. The column was washed with 50 ml CPO lysis buffer, followed by 50 ml CPO lysis buffer containing 20 mM imidazole. Protein elution was accomplished by passing 10 ml of CPO lysis buffer containing 250 mM imidazole (adjusted to pH 7) through the column. Fractions of approximately 1 ml each were collected. The protein concentration of each fraction was determined by the method of Bradford [23]. In all cases, enzyme activities were measured for the supernatant fraction as well as all fractions eluted from the column; activities for these various fractions were comparable for wild-type and mutant enzymes (data not shown).

Substrate preparation

For enzyme assays, three different substrates (coproporphyrinogen-III, harderoporphyrinogen, and mesoporphyrinogen-VI) were used. The first is the authentic substrate, while harderoporphyrinogen is the intermediary monovinyl metabolite (Figure 1A). Mesoporphyrinogen-VI (meso'gen-VI) (Figure 1B) has been previously used to assess the ability of chicken CPO to recognize variations in ring substituents [4,5,24]. Use of the three substrates thus allows for the relative comparison of rates for the first and second oxidative decarboxylation for both wild-type and mutant enzymes. The substrates were prepared from the corresponding porphyrin tetramethyl esters by treatment overnight with 8.3 M HCl and reduction with 3% sodium amalgam as previously reported [5,24]. Coproporphyrin-III tetramethyl ester was purchased from Aldrich Chemical (Milwaukee, WI). Harderoporphyrin trimethyl ester and mesoporphyrin-VI dimethyl ester were synthesized from a,c-biladiene intermediates [24–26]. In all cases, the reduced porphyrinogens were used immediately following reduction and quantitated spectrophotometrically at 406 nm in 8.3 M HCl. For time course experiments, substrates were used in the range of 0.7 to 1 μM as the final concentration in the enzyme incubations, concentrations above the K_m value for coproporphyrinogen III of 0.3 μM reported in this work (Table 1).



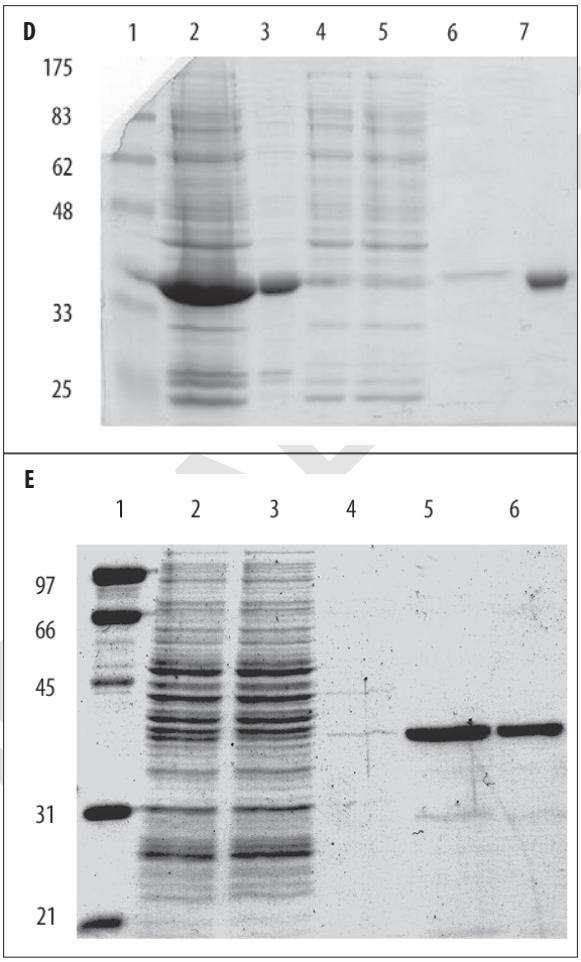


Figure 3. (**A**) Purification of 6x-His-tagged wildtype CPO expressed in *E. coli*. Lane 1: molecular weight standards (kDa), Lane 2: 100,000 × g supernatant (20 mg), Lane 3: metal affinity column non-binding fraction (20 mg), Lanes 4–8: 250 mM imidazole metal affinity column elution (1 µg, 2 µg, 4 µg, 8 µg, and 12 µg, respectively). (**B**) Purification of 6x-His-tagged CPO mutant enzyme H148A expressed in *E. coli*. Lane 1: molecular weight standards (kDa), Lane 2: 100,000 × g supernatant (20 µg), Lane 3: metal affinity column non-binding fraction (20 µg), Lanes 4 and 5: 250 mM imidazole metal affinity column elution (2 µg and 4 µg, respectively). (**C**) Purification of 6x-His-tagged CPO mutant enzyme H158A expressed in *E. coli*. Lane 1: molecular weight standards (kDa), Lane 2: 100,000 × g supernatant (20 µg), Lane 3: metal affinity column non--binding fraction (10 µg), Lanes 4 and 5: 250 mM imidazole metal affinity column elution (2 µg and 4 µg, respectively). (**D**) Purification of 6x-His-tagged CPO mutant enzyme H197A expressed in *E. coli*. Lane 1: molecular weight standards (kDa), Lane 2: whole cell lysate, Lane 3: 100,000 × g pellet, Lane 4: 100,000 × g supernatant (20 µg), Lane 5: metal affinity column non-binding fraction (20 µg), Lanes 6 and 7: 250 mM imidazole metal affinity column elution (0.5 µg and 4 µg, respectively). (**E**) Purification of 6x-His-tagged CPO mutant enzyme H227A expressed in *E. coli*. Lane 1: molecular weight standards (kDa), Lane 2: 100,000 × g supernatant (20 µg), Lane 3: metal affinity column non-binding fraction (20 µg), Lane 4: metal affinity column 20 mM imidazole wash fraction, Lanes 5 and 6: 250 mM imidazole metal affinity column elution (4 µg and 2 µg, respectively).

Enzyme assay

Recombinant human CPO was assayed using the micro procedure reported by Jones et al. [27]. Briefly, purified recombinant human CPO (300 µl of 50 µg/ml) was incubat-

ed with the freshly prepared substrates in the dark at 37°C. For each prepared mutant, both wild-type and mutant enzymes were incubated under the same conditions so that data are reported relative to wild-type activity. For each experiment, time courses for both wild-type and mutant enzymes were performed. Specific activities (nmole product formed/min/mg protein) for the three different substrates were determined under apparent initial velocity conditions. Reactions were stopped by the addition of acetic acid: ethyl acetate (3:7 v/v) and extracted into the ethyl acetate layer as reported previously [27]. The porphyrins were converted to their methyl esters and the substrate and product were separated by HPLC using a partisil silica column and a mobile phase solvent of ethyl acetate: cyclohexane (35:65 v/v for coproporphyrin-III and harderoporphyrin and 3:7 v/v for mesoporphyrin-VI). Eluting porphyrins were detected at 404 nm and percent product calculated from the integrated peak areas. When using coproporphyrinogen-III as substrate, total product was calculated as the sum of the monovinyl and divinyl products. In all cases, results were compared to zero time incubations to correct for background.

Kinetics with wild-type CPO and H158A enzyme

Both the wild-type enzyme and the H158A mutant enzyme were assayed under apparent initial velocity conditions with two different substrates (coproporphyrinogen-III and harderoporphyrinogen) at various concentrations from 0.1 to 2.4 μM. Incubations were done under standard conditions with 7.5 μg enzyme per incubate.

RESULTS

Expression and purification of His-tagged human wild-type (WT) and mutant CPO proteins

To assess the level of endogenous bacterial activity, *E. coli* cultures expressing a non-related recombinant enzyme (*Pseudomonas mevalonii* HMG-CoA reductase) were analyzed. No coproporphyrinogen oxidase activity was detectable in the 250 mM imidazole elution fraction from the metal affinity column (where the recombinant enzyme elutes). Bacterial CPO activity was found in the 100,000 × g supernatant fraction and in the fraction that did not bind to the affinity column. Expression of recombinant wild-type human CPO in *E. coli* increased the activity in the supernatant fraction by approximately 500-fold compared to the level of endogenous bacterial CPO activity in the control culture. For all histidine mutants, CPO enzyme activities in the 250 mM elution fraction were substantially higher than that obtained using the *Pseudomonas mevalonii* HMG-CoA reductase fractions.

The inducible pET expression system (Novagen) resulted in a large yield of recombinant wild-type CPO that was easily purified using affinity chromatography. An SDS-PAGE analysis of purification fractions is shown in Figure 3A. Levels of recombinant wild-type CPO were estimated to be as high as 30% of the total cellular protein. Yields of electrophoretically pure wild-type CPO were as high as 95 mg per liter of bacterial culture. The large amounts of pure CPO obtainable using this expression system should facilitate future studies of human CPO using biophysical methods that require large amounts of pure protein. For all mutants, electrophoretic purity was also evaluated, with results very similar to those shown for the wild-type enzyme with substantial amounts of enzyme found in the soluble fraction and in the 250 mM elution fraction (Figure 3 B–E).

Catalysis by recombinant human CPO (wild-type)

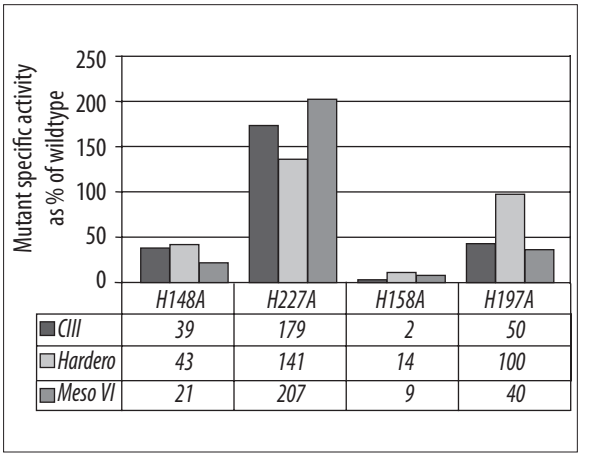
For the human wild-type enzyme, the specific activities (nmole product/min/mg protein) using the three different substrates were 4.9±0.9 for coproporphyrinogen-III, 5.1±1.8 for harderoporphyrinogen, and 1.7±0.7 for mesoporphyrinogen-VI (mean ±SD for n=5). Our data indicate that the mesoporphyrinogen-VI is used much less efficiently by the wild-type enzyme relative to the other two substrates. This is in contrast to the chicken enzyme which apparently uses mesoporphyrinogen-VI and coproporphyrinogen-III with equal ability [24]. Using coproporphyrinogen-III, there is little accumulation of the monovinyl product (harderoporphyrinogen) relative to the divinyl product (protoporphyrinogen-IX) at any incubation time, suggesting that the first decarboxylation is a slower process than the second and thus may be the rate limiting event.

Catalysis by recombinant human CPO mutant enzymes

For the four different histidine mutants, the ability to use the three different substrates is indicated in Figure 4 as percent of wild-type specific activity. Mutant enzyme H148A, though less active relative to wild-type enzyme under identical incubation conditions, still retained 39% of wild-type activity for the overall conversion of coproporphyrinogen-III to protoporphyrinogen-IX. This is similar to the reduced activity seen with harderoporphyrinogen as substrate (43% of wild-type). A decrease in activity was also apparent for the first oxidative decarboxylation using mesoporphyrinogen-VI (21% of wild-type). This decrease in activity relative to wild-type suggests His148 is not directly involved in catalysis but may play a structural role in CPO.

Mutant enzyme H197A also retained the ability to efficiently catalyze the overall reaction (conversion of coproporphyrinogen-III to protoporphyrinogen-IX). The ability to catalyze the overall reaction at only 50% of the wild-type rate is likely due to a decrease in the ability to perform the first oxidative decarboxylation, as estimated utilizing mesoporphyrinogen-VI as substrate. The second oxidative decarboxylation step was not impaired, with mutant enzyme H197A retaining 100% of the wild-type activity using harderoporphyrinogen as substrate.

Interestingly, mutant enzyme H227A catalyzed the conversion of coproporphyrinogen-III to protoporphyrinogen-IX at a rate almost twice that of the wild-type enzyme. The increase in specific activity, relative to wild-type enzyme, of the H227A mutant is illustrated by the ability of the mutant enzyme to use all three substrates better than wild-type enzyme. With the other three mutants, as well as the wild-type human CPO, the first oxidative decarboxylation is the apparent rate limiting step for catalysis. However, for H227A the first oxidative decarboxylation no longer appears to be the rate limiting step when His227 is replaced by alanine since the incubations with mesoporphyrinogen-VI yielded higher specific activities than with either of the other two substrates.



	H148A	H227A	H158A	H197A
CIII	39	179	2	50
Hardero	43	141	14	100
Meso VI	21	207	9	40

Figure 4. A comparison of specific activities of mutant enzymes as percent of wildtype specific activity using three different substrates. Values are the average of 2 measurements.

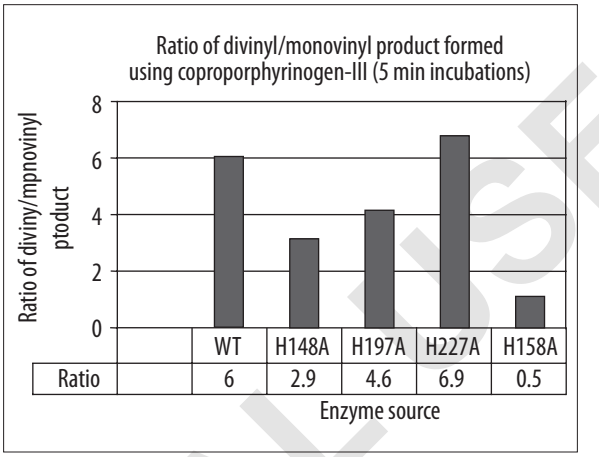


Figure 5. A comparison of the ratio of divinyl product formed to monovinyl product formed using coproporphyrinogen-III as substrate (after 5 min of incubation with 15 ug of enzyme, at 37°C)

The mutant enzyme most catalytically impaired was that for which His 158 was replaced by alanine. The ability of the H158A mutant enzyme to catalyze the conversion of coproporphyrinogen-III to protoporphyrinogen-IX was decreased some 50-fold. This decrease, while large, still reflects considerable enzyme activity, as the purified mutant enzyme retained activity per mg of enzyme approximately 100-fold greater than the baseline value for a control purification procedure conducted on cells expressing an unrelated protein, *Pseudomonas mevalonii* HMG-CoA reductase. The decrease in specific activity was attributable to impairment of both the first and second oxidative decarboxylations, with the first catalyzed at 9% of the wild-type and the second at 14% of the wild-type. In a previous study conducted using recombinant mouse CPO, His158 was proposed to be essential for coordination of a metal ion, most likely Cu^{2+} [19]. Crude cell extracts of bacterial cultures, in which His 158 had been mutated to alanine in mouse CPO, resulted in no detectable activity. Analysis of recombinant human CPO by Medlock and Dailey, however, conclusively determined metal ions were not necessary for catalysis [3].

There was little difference in the K_m value for coproporphyrinogen III for mutant enzyme H158A as compared to wild-type enzyme as shown in Table 1. However, when harderoporphyrinogen was used as substrate, there was an apparent difference in the K_m value of the wild-type enzyme and the mutant. This suggests alanine at position 158 has a modest effect on the ability of CPO to bind substrate. There was, however, a more substantial effect on the k_{cat} value that was especially evident with harderoporphyrinogen as substrate. The catalytic efficiency (k_{cat}/K_m) of the wild-type enzyme was substantially higher than that of the H158A mutant (Table 1) using either substrate.

An interesting consequence of replacing histidine 158 with alanine was the relative ability of CPO to catalyze formation of the monovinyl product (harderoporphyrinogen) relative to the divinyl product protoporphyrinogen IX. In Figure 5 the ratio of divinyl product to monovinyl product using coproporphyrinogen-III is shown for the human wild-type enzyme and compared to the values for the various histidine mutants. The H148A, H197A and H227A mutants all have similar abilities to produce a similar ratio of divinyl to monovinyl product as wild-type. However, the H158A mutant yielded a much different ratio for these two products, producing substantially more of the monovinyl product than the divinyl product. It would be of interest to evaluate these histidine mutants using the A ring isomer coproporphyrinogen-IV (C-IV) for which it was previously shown that increased levels of C-IV may be able to compete with the authentic substrate. Such competition has important implications for clinical porphyrias [28].

Discussion

Location of conserved histidines 148, 197, and 227 in the three-dimensional structure of human CPO

During completion of the work described here, three-dimensional structures of both *Saccharomyces cerevisiae* [29] and *Homo sapiens* [30] coproporphyrinogen oxidase were determined. In each case, the structure of CPO was reported to be dimeric. Phillips et al. [29] report the yeast CPO active-site is likely a deep cleft lined by conserved amino acid residues and each monomer has its own active-site. His158 of human CPO appears to be His133 of yeast CPO (in the S5 secondary structural region). The authors locate this region as perhaps one side of the active site cleft. In human CPO [30], each monomer contains a central 7-stranded antiparallel beta sheet with multiple helices present on each side of the sheet. A molecule of citrate is present in the crystallized enzyme, occupying a cleft near the dimer interface. The coordinate file for the CPO monomer (PDB: 2AEX) was downloaded from the protein database and analyzed, allowing us the opportunity to probe the structural basis for our observations upon mutagenesis of the conserved histidines. Two of the conserved histidines mutated in this study (His148 and His197) are present within the interior of the monomer. His148 is found at the C-terminal end of β4 of the central seven-stranded beta sheet while His197 is in the middle of α5, projecting from this alpha helix into the interior of the monomer. His148 and His 197, therefore, form a variety of atomic interactions that are likely to play a role in maintaining the structural integrity of the monomer structure. These observations support

our data that His148 and His197 are not involved in catalysis. Histidine 227 is the only one of the four conserved histidines investigated in this study that is present on the surface of the monomer, and is thought to be involved in monomer-monomer interaction at the dimerization surface in addition to the atomic interactions within a single monomer.

Location of histidine 158 in the three-dimensional structure of human CPO and possible role in catalysis

Two recent catalytic mechanisms for CPO have proposed a base-catalyzed oxidative decarboxylation [30,31]. Though the mechanisms differ with respect to the proton abstracted and the nature of the intermediates, each requires an amino acid that acts as a base in the active site. His158, present at the N-terminal end of β5 of the seven-stranded beta sheet, is present in the putative active site electrostatic cleft containing a citrate molecule. The proximity of His158 to citrate led to the proposal that this amino acid may perform an essential catalytic function, possibly as a base to abstract a proton from coproporphyrinogen III, generating a carbanion [30] or a pyrrolic anion [31]. The prediction that His158 is present in the active site of CPO is supported by our data, since H158A retained less than 5% of the wild-type activity. However, the significant remaining activity in H158A suggests that His158, though important for catalysis, is not essential. Our observation that the H158A mutant enzyme retains significant activity is contrary to Kohno et al. [19] in which the H158A mutant of mouse CPO was reported to have no activity. This discrepancy may be due to the inability of Kohno et al. to detect the low level of activity since enzymes were assayed in unpurified cytosolic fractions, not as purified, highly concentrated samples. In our case we had to add a significantly greater quantity of H158A enzyme to each assay to reliably measure activity, made possible by a highly purified, concentrated enzyme sample. In addition, Kohno et al. showed that mouse CPO contains copper and His158 was directly implicated in copper coordination since mouse CPO mutant enzyme H158A displayed a very low copper content relative to wild-type enzyme. In contrast to the mouse enzyme, human CPO has been shown to not be a metalloenzyme [3], therefore His158 does not function to coordinate copper.

Conclusions

Our data show that mutant forms of human coproporphyrinogen oxidase, where a conserved histidine residue at either position 148, 158, 197, or 227 is replaced by alanine, are still catalytically competent. The level of activity remaining in the mutant forms of this enzyme is several orders of magnitude greater than expected if only the histidine amino acid side chain was directly participating in catalysis. Therefore, we conclude that these conserved histidine residues are not absolutely essential for the catalytic mechanism of human coproporphyrinogen oxidase but are likely to be important in maintaining the native conformation. The large decrease in activity of the H158A mutant enzyme supports the hypothesis that this amino acid is at or near the active site of the enzyme, possibly reflecting an important role in maintaining the active site architecture of the native enzyme. However, until the crystal structure of CPO has been solved as an enzyme-substrate complex, the roles of active site amino acids remain ambiguous.

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