Human Coproporphyrinogen Oxidase Is Not a Metalloprotein*

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Coproprophyrinogen oxidase (CPO) (EC 1.3.3.3), the antepenultimate enzyme in the heme biosynthetic pathway, catalyzes the conversion of coproporphyrinogen III to protoporphyrin IX. Previously, based upon metal analysis and site-directed mutagenesis of purified recombinant enzyme, it has been suggested that CPO contains and requires copper for activity (Kohno, H., Furukawa, T., Tokunaga, R., Taketani, S., and Yoshinaga, T. (1996) Biochim. Biophys. Acta 1292, 156–162). To examine this putative metal site in human CPO, the cDNA encoding human CPO was engineered into an expression vector with a His6 tag at its amino terminus, and the protein was expressed in Escherichia coli and purified to apparent homogeneity using nickel-nitroliotriacetic acid affinity chromatography. Activity of the purified protein was monitored by a coupled fluorometric assay that employed purified protoporphyrinogen oxidase to convert protoporphyrinogen to protoporphyrin, thereby allowing the direct fluorescence determination of protoporphyrin IX produced. CPO has an apparent $K_m$ of 0.6 $\mu M$ and an apparent $K_{cat}$ of 16 min$^{-1}$ with coproporphyrinogen III as substrate. Metal analysis of the enzyme was carried out via ultraviolet and visible spectroscopy, inductively coupled plasma atomic emission spectroscopy, and electron paramagnetic resonance spectroscopy. The data presented demonstrate that human CPO contains no metal center, that it is not stimulated in vitro by iron or copper, and that addition of these metals to cultures expressing the protein has no effect.

Coproporphyrinogen oxidase (CPO)$^\dagger$ (EC 1.3.3.3) catalyzes the antepenultimate step in the heme biosynthetic pathway, the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX. In this reaction two molecules of $O_2$ are consumed, and two molecules CO$_2$ and two protons are released in the conversion of the 2- and 4-propinyl groups to vinyl groups (1). CPO has been cloned and its DNA sequence has been determined for a number of prokaryotic and eukaryotic organisms including, yeast (2), soybean (3), Salmonella typhimurium (4), Escherichia coli (5), mouse, and human (7, 8). The enzyme has been purified from several sources including bovine liver (9), mouse liver (10), yeast (11), and mouse (recombinant) (12). The enzyme is believed to be located in the intermembrane space of the mitochondria associated with the outer surface of the inner membrane (13).

Deficiency in this enzyme in humans leads to hereditary coproporphyria (HCP). This disorder is an autosomal dominant disease. In most cases of HCP, CPO activity is reduced to approximately 50%, which leads to excretion of coproporphyrin in urine and stool. Symptoms of the disease include neurological disturbances and cutaneous photosensitivity (14). Recent sequencing of the human CPO gene has allowed the identification of mutations leading to HCP. From the primary sequence of the protein, these mutations appear to be diverse. Two such mutations are a glycine to serine near the NH$_2$ terminus (15) and an arginine to tryptophan near the COOH terminus (16). Two reports have suggested that CPO from yeast (11) and mouse (12) may contain a metal atom that may aid in the reaction catalyzed by CPO. Both iron and copper have been suggested as cofactors. In the work reported below we describe a new purification and assay method and investigate the metal content of purified recombinant human CPO using a variety of spectroscopic techniques. The data presented clearly show that CPO does not contain either iron or copper as previously suggested and that these metals do not stimulate the enzyme activity.

**MATERIALS AND METHODS**

**Plasmid Construction**—CPO was cloned from a cDNA human placental library (17) using PCR. Primers were designed to recover the cDNA encoding CPO but lacking the mitochondrial targeting sequence. The sense primer used was 5'-CATGGGCCCCGGCTGTGACCGA-3', and the antisense primer was 5'-ATTCTGGCTGATCAACGGC-3'. PCR reaction mixtures contained 1 $\mu g$ of human placental cDNA library as template, 1 $\mu g$ of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl$_2$, 0.2 mM of each dNTP, and 5 units of Taq Polymerase (Fischer) in a final reaction volume of 100 $\mu l$. The reaction mixture was denatured for 5 min at 95 °C and amplified for 30 cycles (1 min at 95 °C, 1 min at 54 °C, and 2 min at 72 °C) in a DNA thermocycler (Perkin-Elmer). A 1000-base pair cDNA fragment was obtained and isolated by agarose gel electrophoresis followed by extraction with Geneclean (BIO101). A second PCR reaction was set up using the first PCR product as template DNA and 1 $\mu g$ of each primer, 10 mM Tris-HCL, pH 8.3, 50 mM KC1, 2 mM MgCl$_2$, 0.2 mM of each dNTP, and 5 units of Taq Polymerase (Fischer) in a final reaction volume of 100 $\mu l$. The reaction mixture was denatured for 5 min at 95 °C and amplified for 30 cycles (1 min at 95 °C, 1 min at 54 °C, and 2 min at 72 °C) in a DNA thermocycler (Perkin-Elmer). A 1000-base pair cDNA fragment was obtained and isolated by agarose gel electrophoresis followed by extraction with Geneclean (BIO101). A second PCR reaction was set up using the same components as in the first reaction, except 1 $\mu g$ of the first PCR product was used as the template DNA and 1 $\mu g$ of each of the primers listed above containing the appropriate restriction sites and a His$_6$ tag at the NH$_2$ terminus. The sense primer, 5'-CCATGGGCTCAC- CATCACCATCATCACATCGTGTGACCGA-3', was used to incorporate a Ncol site (underlined), whereas the antisense primer, 5'-AAGCTTAT- TCTGCGTGTGACCGA-3', was used to incorporate a HindIII site (underlined) at the 3' end of the PCR product. The second PCR reaction was set up using the same components as in the first reaction, except 1 $\mu g$ of the first PCR product was used as the template DNA and 1 $\mu g$ of each of the primers listed above containing the appropriate restriction sites were used. The thermocycling procedure from the first PCR reaction was used, but the annealing temperature was changed to 52 °C. A 1000-base pair piece of DNA was obtained and identified by agarose gel electrophoresis. The band was excised from the gel and cDNA isolated using the Wizard PCR Prep (Promega). The cleaned fragment was then cloned into the plasmid, pCR II, with the TA cloning Kit (Invitrogen). Blue white colony screening was used to select colonies containing the plasmid with inserted cDNA fragment. Plasmid DNA was purified
using Wizard Minipreps (Promega), and a double digest to excise the CPO cDNA was done on the plasmid DNA with NcoI and HindIII. This fragment was ligated into a modified pBTac expression plasmid (18) that had been digested with NcoI and HindIII. The resulting CPO expression vector was named pHHCPO.

Expression and Purification—One-liter cultures of Circlegrov media (BIO101) with 100 mg of ampicillin were inoculated with E. coli JM109 that had been transformed with pHHCP. Cultures were grown at 37 °C for 18–20 h with 250 rpm shaking. Cells were harvested by centrifuging for 20 min at 10,000 × g at 4 °C. Cell pellets were resuspended in 60 ml resuspension buffer, consisting of 50 mM NaH2PO4, 300 mM NaCl, 0.5% n-octyl-β-D-glucopyranoside, 1 mM phenylmethylsulfonyl fluoride, and 100 mM Tris-HCl, pH 8.0. Resuspended cells were sonicated four times for 30 s each time and then centrifuged at 100,000 × g for 60 min at 4 °C. This separated the solubilized protein fraction from the membranes. Five milliliters of nickel-NTA resin (Qiagen) were placed into a column and equilibrated at 25 °C with 50 ml of the resuspension buffer used above. The soluble protein fraction was then loaded on the column. The column was washed with 50 ml of resuspension buffer plus 20 mM imidazole, pH 7.0. Following this, CPO was eluted with resuspension buffer plus 250 mM imidazole, pH 7.0, and 500–µl fractions were collected.

Protein purity was assessed by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (19) and UV-visible spectra (Cary-219 Spectrophotometer). Protein concentration of cell lysate, column flow through, and column wash was determined with bicinchoninic acid (20) reagent from Pierce, using bovine serum albumin as the standard.

Assay—A coupled fluorometric assay similar to that described by Labbe et al. (21) was designed to assay purified CPO. Recombinant Myxobacterium xanthus PPO purified as described by Dailey and Dailey (18) was used in the assay. Buffer used for the assay consisted of 0.1 x NaH2PO4, pH 8.0, 0.2% Tween 20, and 2.5 mM glutathione. Assay reactions consisted of assay buffer, purified CPO, purified PPO, and substrate in a total volume of 1.1 ml. For all activity measurements purified bacterial PPO was used in a 3-fold molar excess of CPO. In some assays Cu2+ was added in an amount equimolar to CPO. All assay reaction measurements were carried out at 37 °C in a dark room. 100-µl aliquots from assay mixtures were removed and read at 0, 10, 20, and 30 min after addition of substrate. Fluorometric measurements of the aliquots were taken by diluting them in 1 ml of a 1:10 dilution of assay buffer. A Perkin-Elmer 650–40 fluorescence spectrophotometer with excitation wavelength at 405 nm and emission wavelength at 635 nm was used to follow the reaction. Substrate coproporphyrinogen III was prepared by reducing coproporphyrinogen III dihydrolchloride (Porphyrin products, Logan, UT) in a dimly light room with 3% sodium amalgam. Substrate was quantitated following autoxidation to coproporphyrin III on the bench top for 2–3 days. It was then diluted 1:100 in 0.1 N HCl, and its absorbance was measured at 400 nm using a molar extinction coefficient of 489,000 (22).

Metal Analysis—Purified recombinant CPO was analyzed for the presence of metal ions by inductively coupled plasma atomic emission spectroscopy (ICP) at the University of Georgia Chemical Analysis Lab. ICP metal analysis was done on four separate samples with enzyme protein concentration ranging from 40 to 200 µg. With one sample an internal copper standard was included. Electron paramagnetic resonance spectroscopy of purified CPO was carried out on both oxidized and dithionite reduced samples. These data were compared with those of Fe2+ and Cu2+. Also the spectrum of Cu2+ added to purified CPO was measured, and this spectrum was compared with that of Cu2+ in solution to see if there was any change due to Cu2+ binding to CPO.

RESULTS AND DISCUSSION

Purification—in this paper we describe an improved purification and assay procedure for recombinant human CPO. The purification method first utilizes the addition of a His6 tag at the NH2 terminus of human CPO. After expression in E. coli JM109, the protein can be rapidly purified by using a nickel-NTA column. This procedure yields 8–15 mg of purified protein from 1 liter of culture. This purification scheme produces a large amount of purified CPO in a relatively short time compared with previously described methods (12). SDS-polyacrylamide gel electrophoresis of the column fractions documents the purification of recombinant CPO from the cell lysate (Fig. 1). The purified protein appears as a single band corresponding to a molecular weight of 37,000.

UV-visible spectrum of purified CPO has absorption at λmax of 280 nm with a small feature at λ408 nm (Fig. 2). The absorption at 280 nm was used to quantitate the protein using an estimated molar extinction coefficient of 52,000 as determined by Peptidesort (23) using the published amino acid sequence (7, 8). The absorption band at 406 nm was determined by fluorescence emission and excitation scans to be due to a small amount of contaminating porphyrin (data not shown).

Assay—The improved assay of CPO utilizes a pure preparation of bacterial PPO in a coupled assay similar to that described by Labbe et al. (21). The production of protoporphyrin IX is followed directly by fluorescence measurements using 405 nm as the excitation wavelength and 635 nm as the emission wavelength. The relative fluorescence units obtained are converted to micromoles of protoporphyrin IX produced using a standard curve. Data obtained from assays in which the amount of CPO and PPO were varied demonstrated that PPO activity in a 3-fold molar excess over CPO are sufficient to...
completely and rapidly convert all porphyrinogen to porphyrin. Under these conditions, the production of protoporphyrin IX is linear with respect to both CPO concentration and time. The kinetic data obtained using this assay yield an apparent $K_m$ of 0.6 $\mu$M for coproporphyrinogen III and an apparent $K_{cat}$ of 16 min$^{-1}$ under atmospheric concentrations of oxygen (Fig. 3). The $K_m$ obtained is in agreement with that obtained from normal human liver by Grandchamp and Nordmann (24) using a radiolabeled assay and with other $K_m$ values reported in the literature, which range from 0.05 to 47 $\mu$M. Advantages of the assay described above are that no radioactive material is used and that the PPO preparation is pure and contains no contaminating proteins that may affect CPO or alter the amount of final product.

**Metal Analysis**—Previous reports suggested that a metal atom may be involved in the reaction catalyzed by CPO (11, 12) and examination of known sequences reveals the presence of several conserved histidine, aspartate, glutamate, and cysteine residues that could be involved in the metal ligation by the enzyme (Fig. 4). Yeast CPO was reported as having two iron atoms per homodimer (11), whereas more recently Kohno et al. (12) reported that purified recombinant mouse CPO contain one copper atom per polypeptide. However, our initial purification yielded no evidence of metal in CPO. The UV-visible spectrum revealed no evidence for a chromophoric metal, but because of the previous reports we were concerned that CPO associated metal may have been lost during purification or that our metal analysis was insensitive.

To approach the question of the metal loss we rationalized that metal loss possibly occurring on the elution from the NTA column with imidazole would be reflected in loss of enzyme activity. However, as the data in Table I clearly demonstrate, the overall recovery of activity in all fractions is essentially 100%. These results are highly reproducible. In addition, the inclusion of copper in the growth medium had no effect on enzyme recovery. Although $K_{cat}$ values for the purified native human CPO have not been reported, the $K_{cat}$ reported herein is comparable with that of purified native mouse liver CPO (11). Although the previous authors did not report an apparent $K_{cat}$ but only a specific activity, one would estimate that our reported $K_{cat}$ is approximately 4-fold higher than that of native mouse.

It is unlikely that our metal analysis (Table II) was insensitive because the samples analyzed by ICP were active enzyme at a concentration of up to 200 $\mu$M. Samples spiked with a

![Fig. 3. Lineweaver-Burk plot of purified human coproporphyrinogen oxidase activity versus coproporphyrinogen III concentration. Activity (v) is expressed as nmol of protoporphyrin IX produced per min$^{-1}$ pmol$^{-1}$ of enzyme at 37°C.](image)

![Fig. 4. Amino acid sequence comparison of five aerobic coproporphyrinogen oxidases. The alignment was created by the Genetics Computer Group program Pileup (23). Conserved residues in all five sequences are denoted underneath with an asterisk. Swiss Prot accession numbers are: human, P36551; mouse, P36532; yeast, P11353; soybean, P36053; E. coli, P98553; S. typhimurium, P33771.](image)
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Purification of recombinant human CPO

| Fraction          | Total Protein | Total activity | Specific activity | Recovery |
|-------------------|---------------|---------------|------------------|----------|
| mg                | units         | units/mg      | %                |          |
| Cell Lysate       | 600           | 7300          | 12.2             | 100      |
| Column flow through | 511           | 1400          | 2.6              | 19       |
| Wash              | 34            | 2900          | 86               | 40       |
| Purified protein  | 12.3          | 3100          | 258              | 43       |

* Activity is expressed in nmol of protoporphyrin IX produced per minute at 37 °C.

Metal analysis of purified recombinant human CPO

| Metal         | Average ppm | Content mol metal/mol enzyme |
|---------------|-------------|------------------------------|
| Copper        | 0.0510      | <0.01                        |
| Iron          | 0.1543      | <0.01                        |
| Magnesium     | 0.0983      | <0.01                        |
| Nickel        | 0.2509      | <0.01                        |
| Zinc          | 1.2997      | <0.01                        |

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Fig. 5. Electron paramagnetic resonance spectroscopy. EPR spectroscopy was done on the purified recombinant enzyme, both oxidized (top) and reduced (middle). This was compared with the signal obtained with both Cu(II) (bottom) and Fe(III) (data not shown). Concentrations of CPO samples and Cu(II) were 50 μM. All spectra are shown on the same scale. In the purified enzyme sample the only signal seen was a small signal at approximately 3350 Gauss, characteristic of the empty cavity. Spectra were recorded at 9 K, with microwave power of 1 mW, modulation amplitude of 0.102 mT, and microwave frequency of 9.596 GHz.

stoichiometric amount of copper or iron yielded unequivocal results. Likewise, EPR spectroscopy showed that no paramagnetic metals such as Fe(II) and Cu(II) were present in the active purified protein. Spectra of both reduced and oxidized samples were taken, and neither contained an active EPR signal (Fig. 5). EPR spectra with broader sweep width showed no signal at g = 4.3, which would be characteristic of high spin ferric iron (data not shown). Also, purified protein samples were incubated with equimolar amounts of CuCl2 and assayed for changes in CPO activity. No change in activity was noted upon a 48-h incubation with Cu(II) (data not shown). These results indicated that a metal atom is not involved in the reaction catalyzed by human CPO.

Absence of a metal atom or other cofactor still leave the question of the reaction mechanism unanswered. Yoshinaga and Sano (25) suggested that the reaction occurs through β-hydroxypropionate formation in which tyrosyl residues are involved. They demonstrated that modification of two tyrosine residues results in inactivation of the enzyme. From sequence alignment of three eukaryotic sources, yeast, mouse, and human, eight conserved tyrosine residues may be useful in elucidating the active site and the overall reaction mechanism.

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