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Human protoporphyrinogen oxidase: Expression, purification, and characterization of the cloned enzyme

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Abstract

Protoporphyrinogen oxidase (E.C.1.3.3.4) catalyzes the oxygen-dependent oxidation of protoporphyrinogen IX to protoporphyrin IX. The enzyme from human placenta has been cloned, sequenced, expressed in *Escherichia coli*, purified to homogeneity, and characterized. Northern blot analysis of eight different human tissues show evidence for only a single transcript in all tissue types and the size of this transcript is approximately 1.8 kb. The human cDNA has been inserted into an expression vector for *E. coli* and the protein produced at high levels in these cells. The protein is found in both membrane and cytoplasmic fractions. The enzyme was purified to homogeneity in the presence of detergents using a metal chelate affinity column. The purified protein is a homodimer composed of subunits of a molecular weight of 51,000. The enzyme contains one noncovalently bound FAD per dimer, has a monomer extinction coefficient of 48,000 at 270 nm and contains no detectable redox active metals. The apparent K_m and K_{cat} for protoporphyrinogen IX are 1.7 μM and 10.5 min^{-1}, respectively. The enzyme does not use coproporphyrinogen III as a substrate and is inhibited by micromolar concentrations of the herbicide acifluorfen. Protein database searches reveal significant homology between protoporphyrinogen oxidase and monoamine oxidase.

Keywords: heme biosynthesis sequence comparison, protoporphyrinogen oxidase

The six-electron oxidation of protoporphyrinogen IX to the planar, fully conjugated macrocycle protoporphyrin IX is catalyzed by the enzyme protoporphyrinogen oxidase (E.C.1.3.3.4) (Porra & Falk, 1964; Poulson & Polglase, 1975; Dailey, 1990). In eukaryotic cells this enzyme is located on the cytosolic side of the inner mitochondrial membrane and requires molecular oxygen for activity (Deybach et al., 1985; Ferreira et al., 1988; Dailey, 1990). The protein has been purified to apparent homogeneity from mouse (Dailey & Karr, 1987) and bovine (Siepker et al., 1987) liver, and from yeast mitochondria (Camadro et al., 1994), and in all instances it was reported to contain a flavin cofactor and requires detergent to solubilize the protein from the membrane. However, because the enzyme is present in low concentrations in cells, the amount of isolated purified protein has been too low for proper biochemical and biophysical characterization. Recently, two oxygen-dependent bacterial protoporphyrinogen oxidases have been cloned and expressed (Hansson & Hederstedt, 1992, 1994; Dailey et al., 1994; H.A. Dailey & T.A. Dailey, in prep.) and the cDNA for mouse (Dailey et al., 1995) and human (Dailey & Dailey, 1995; Nishimura et al., 1995) have been cloned and sequenced. Based upon the derived amino acid sequences, it is clear that all share certain structural characteristics such as molecular size and the presence of a dinucleotide binding motif.

Interest in the mammalian protoporphyrinogen oxidase arises from both its biochemical and catalytic characteristics (i.e., it catalyzes a six-electron oxidation), and its biomedical significance. In humans, the dominantly inherited genetic disease variegate porphyria (VP) is attributable to decreased levels of the enzyme (Brenner & Bloomer, 1980a; Deybach et al., 1981). This disorder, whose symptoms include both chronic photodermatitis and intermittent neurovisceral episodes, affects approximately 10,000 individuals in South Africa and an undetermined number in other countries of the world (Day, 1986). The symptomology of VP, along with its highly variable penetrance in affected individuals, makes the study of the nature of the enzyme causing the disease of great interest.

An additional, and more recently recognized, area of interest has to do with the fact that a large class of herbicides has been shown to act by inhibiting protoporphyrinogen oxidase of plants

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both in vitro and in vivo (Matringe et al., 1989; Jacobs et al., 1991). Studies on some of these herbicides in cell culture suggest that exposure of animals to these compounds may profoundly affect the exposed animal (Jacobs et al., 1992).

In the current study, we have, for the first time, expressed the cloned human protoporphyrinogen oxidase in *Escherichia coli* so that milligram quantities of the enzyme are produced. The purified recombinant enzyme was characterized and shown to be a dimeric, FAD-containing oxidase, which, in several aspects, resembles mammalian mitochondrial monoamine oxidase.

Results

Cloning and sequence of human protoporphyrinogen oxidase

Cloning of the enzyme was accomplished by using functional complementation of *E. coli* SASX38 (Sasarman et al., 1993) with a rescued λZAP phagemid human placental library in a procedure identical to one used previously by this lab to isolate the murine enzyme cDNA. Use of the rescued plasmid rather than a λ library was necessary because the SASX38 cells do not behave as Hfr^+ cells. From a screening of approximately 3×10^5 library members, a single positive clone was isolated. The nucleotide sequence is shown in Figure 1. Northern blot analysis of RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas demonstrated the presence of a single transcript of approximately 1.8 kb (Fig. 2).

Expression and purification of the recombinant human enzyme

For expression and purification purposes, the human cDNA was modified so that a His_6 tag was placed upstream from the ATG start site. Because it was found that insertion of the His_6 tag immediately adjacent to the ATG start codon resulted in an expressed protein that bound poorly to the Ni^{+2} chelate column, a spacer consisting of nine amino acids was added to the front of the protein. This had no discernable affect on the expressed enzyme. The engineered cDNA was inserted into the unique *Nhe* I site of the expression vector pTrcHis B. The resultant expression vector is named pHPPO-X and it is maintained in *E. coli* JM109.

To express recombinant human protoporphyrinogen oxidase, transformed cells were grown in Circlegrow (BIO101, Inc.) for 18–20 h. No induction scheme was followed because it was found that growth into stationary phase was sufficient for optimal induction. The Ni^{+2} chelate affinity column purification scheme yielded 5–10 mg of protein from a 1-L culture. SDS-PAGE (Fig. 3) shows the presence of a single protein band with molecular weight of 51,000. The purified enzyme is stable for days at 4 °C, but does appear to be slowly proteolyzed to lower molecular weight fragments.

Characteristics of mammalian protoporphyrinogen oxidase

Gel filtration of the purified enzyme on a Sephacryl S-300 column in the presence of 0.2% *n*-octyl-β-D-glucopyranoside detergent results in the elution of all enzyme in a fraction corresponding to a molecular size of about 100 kDa. No peak at 50 kDa is detected. These data are consistent with the active enzyme being a homodimer.

Visible/ultraviolet spectroscopy of the purified enzyme has the characteristic spectrum the oxidized FAD cofactor (Fig. 4). Based upon amino acid content of the protein, the extinction coefficient is 48,000 at 270 nm. Interestingly, enzyme preparations routinely contain only 0.5 molar equivalent of FAD per enzyme monomer and the addition of commercially obtained FAD to the assay reaction mixture did not stimulate activity. The visible spectrum also has a small feature at about 410 nm that is due to some residual product (protoporphyrin) that is present in variable amounts in all enzyme preparations examined. The

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-191  CGGAGAGTAGGAGAGACCGAAAAGGCTGGGGGTGGGAGTAGCGGATTTGAAGCACTTGTT   -132
-131  GGCCTACAGAGGTGTGGCAAGCAGAGCACCTCAGAACTCAGTCGTACTGCCCGCCGCCCG   -72
 -71  AGCCGTGCGAGGGCCGATAGCGAGGGTGTGGCCCTTATCTGCACCCAGCAGAGCGCCGGC   -12
 -11  GGGGTTTCCGCATGGGCCGGACCGTGGTCGTGCTGGGCGGAGGCATCAGCGGCTTGGCCG    48
                M  G  R  T  V  V  V  L  G  G  G  I  S  G  L  A  A     17

  49  CCAGTTACCACCTGAGCCGGGCCCCCTGCCCCCCTAAGGTGGTCCTAGTGGAGAGCAGTG   108
  18    S  Y  H  L  S  R  A  P  C  P  P  K  V  V  L  V  E  S  S  E   37

 109  AGCGTCTGGGAGGCTGGATTCGCTCCGTTCGAGGCCCTAATGGTGCTATCTTTGAGCTTG   168
  38    R  L  G  G  W  I  R  S  V  R  G  P  N  G  A  I  F  E  L  G   57

 169  GACCTCGGGGAATTAGGCCAGCGGGAGCCCTAGGGGCCCGGACCTTGCTCCTGGTTTCTG   228
  58    P  R  G  I  R  P  A  G  A  L  G  A  R  T  L  L  L  V  S  E   77

 229  AGCTTGGCTTGGATTCAGAAGTGCTGCCTGTCCGGGGAGACCACCCAGCTGCCCAGAACA   288
  78    L  G  L  D  S  E  V  L  P  V  R  G  D  H  P  A  A  Q  N  R   97

 289  GGTTCCTCTACGTGGGCGGTGCCCTGCATGCCCTACCCACTGGCCTCAGGGGGCTACTCC   348
  98    F  L  Y  V  G  G  A  L  H  A  L  P  T  G  L  R  G  L  L  R  117

 349  GCCCTTCACCCCCCTTCTCCAAACCTCTGTTTTGGGCTGGGCTGAGGGAGCTGACCAAGC   408
 118    P  S  P  P  F  S  K  P  L  F  W  A  G  L  R  E  L  T  K  P  137

 409  CCCGGGGCAAAGAGCCTGATGAGACTGTGCACAGTTTTGCCCAGCGCCGCCTTGGACCTG   468
 138    R  G  K  E  P  D  E  T  V  H  S  F  A  Q  R  R  L  G  P  E  157

 469  AGGTGGCGTCTCTAGCCATGGACAGTCTCTGCCGTGGAGTGTTTGCAGGCAACAGCCGTG   528
 158    V  A  S  L  A  M  D  S  L  C  R  G  V  F  A  G  N  S  R  E  177

 529  AGCTCAGCATCAGGTCCTGCTTTCCCAGTCTCTTCCAAGCTGAGCAAACCCATCGTTCCA   588
 178    L  S  I  R  S  C  F  P  S  L  F  Q  A  E  Q  T  H  R  S  I  197

 589  TATTACTGGGCCTGCTGCTGGGGGCAGGGCGGACCCCACAGCCAGACTCAGCACTCATTC   648
 198    L  L  G  L  L  L  G  A  G  R  T  P  Q  P  D  S  A  L  I  R  217

 649  GCCAGGCCTTGGCTGAGCGCTGGAGCCAGTGGTCACTTCGTGGAGGTCTAGAGATGTTGC   708
 218    Q  A  L  A  E  R  W  S  Q  W  S  L  R  G  G  L  E  M  L  P  237

 709  CTCAGGCCCTTGAAACCCACCTGACTAGTAGGGGGGTCAGTGTTCTCAGAGGCCAGCCGG   768
 238    Q  A  L  E  T  H  L  T  S  R  G  V  S  V  L  R  G  Q  P  V  257

 769  TCTGTGGGCTCAGCCTCCAGGCAGAAGGGCGCTGGAAGGTATCTCTAAGGGACAGCAGTC   828
 258    C  G  L  S  L  Q  A  E  G  R  W  K  V  S  L  R  D  S  S  L  277

 829  TGGAGGCTGACCACGTTATTAGTGCCATTCCAGCTTCAGTGCTCAGTGAGCTGCTCCCTG   888
 278    E  A  D  H  V  I  S  A  I  P  A  S  V  L  S  E  L  L  P  A  297

 889  CTGAGGCTGCCCCTCTGGCTCGTGCCCTGAGTGCCATCACTGCAGTGTCTGTAGCTGTGG   948
 298    E  A  A  P  L  A  R  A  L  S  A  I  T  A  V  S  V  A  V  V  317

 949  TGAATCTGCAGTACCAAGGAGCCCATCTGCCTGTCCAGGGATTTGGACATTTGGTGCCAT  1008
 318    N  L  Q  Y  Q  G  A  H  L  P  V  Q  G  F  G  H  L  V  P  S  337

1009  CTTCAGAAGATCCAGGAGTCCTGGGAATCGTGTATGACTCAGTTGCTTTCCCTGAGCAGG  1068
 338    S  E  D  P  G  V  L  G  I  V  Y  D  S  V  A  F  P  E  Q  D  357

1069  ACGGGAGCCCCCCTGGCCTCAGAGTGACTGTGATGCTGGGAGGTTCCTGGTTACAGACAC  1128
 358    G  S  P  P  G  L  R  V  T  V  M  L  G  G  S  W  L  Q  T  L  377

1129  TGGAGGCTAGTGGCTGTGTCTTATCTCAGGAGCTGTTTCAACAGCGGGCCCAGGAAGCAG  1188
 378    E  A  S  G  C  V  L  S  Q  E  L  F  Q  Q  R  A  Q  E  A  A  397

1189  CTGCTACACAATTAGGACTGAAGGAGATGCCGAGCCACTGCTTGGTCCATCTACACAAGA  1248
 398    A  T  Q  L  G  L  K  E  M  P  S  H  C  L  V  H  L  H  K  N  417

1249  ACTGCATTCCCCAGTATACACTAGGTCACTGGCAAAAACTAGAGTCAGCTAGGCAATTCC  1308
 418    C  I  P  Q  Y  T  L  G  H  W  Q  K  L  E  S  A  R  Q  F  L  437

1309  TGACTGCTCACAGGTTGCCCCTGACTCTGGCTGGAGCCTCCTATGAGGGAGTTGCTGTTA  1368
 438    T  A  H  R  L  P  L  T  L  A  G  A  S  Y  E  G  V  A  V  N  457

1369  ATGACTGTATAGAGAGTGGGCGCCAGGCAGCAGTCAGTGTCCTGGGCACAGAACCTAACA  1428
 458    D  C  I  E  S  G  R  Q  A  A  V  S  V  L  G  T  E  P  N  S  477

1429  GCTGATCCCCAACTCTCATTCATGAAAATAAAAATTGCTGGAGCTTGAAAAAAAAAAAAA  1488
 478    *                                                           497

1489  AAAAA  1493
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Fig. 1. Nucleotide sequence of human protoporphyrinogen oxidase cDNA. The putative translation product is shown below the nucleotide sequence.

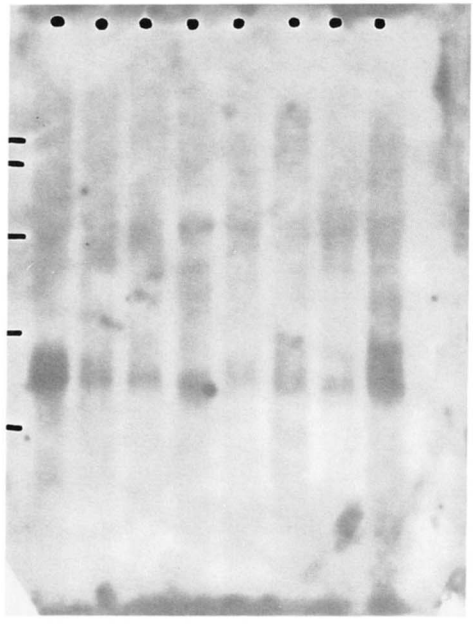

Fig. 2. Northern blot analysis of human tissue mRNA. See text for details of the procedure. The blot was obtained from Clontech. Tissues shown (l to r) are human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Molecular weight markers on the left side are 9.5, 7.5, 4.4, 2.4, and 1.35 kb.

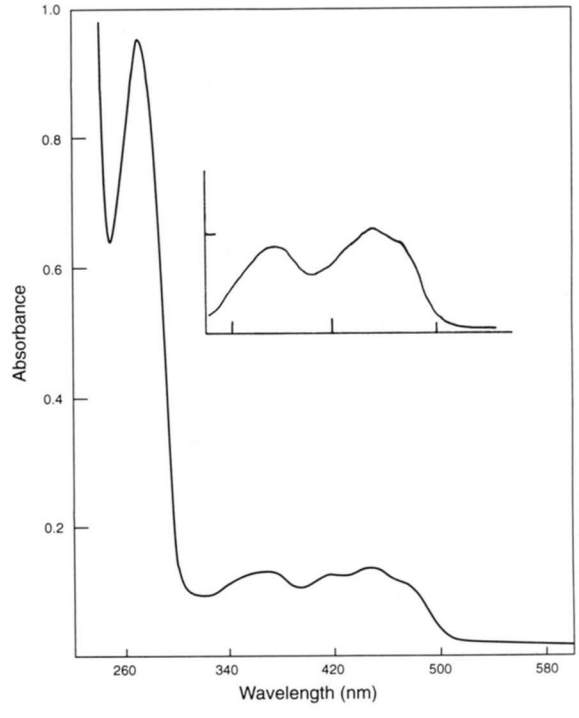

Fig. 4. Visible/ultraviolet spectrum of human protoporphyrinogen oxidase. The enzyme sample (18 μM) was in 20 mM Na phosphate pH 7.4, 0.2% *n*-octyl-β-D-glucopyranoside. Inset: the spectrum of the acid-extracted FAD obtained from the enzyme preparation.

intensity of the 410 band, which is obscured by the flavin absorbance, indicates that there is less than 1% (mol protoporphyrin 1 mol enzyme) contamination present. The flavin present in the enzyme is tightly associated, but is not covalently bound. Acid, organic solvents, or 1% SDS will release the flavin into solution. Five percent trichloroacetic acid precipitates the protein and releases FAD into solution (Fig. 4). FAD was identified by its pH dependent fluorescence intensity (Koziol, 1971) (data not shown).

The purified enzyme was found to use protoporphyrinogen IX, but not coproporphyrinogen III, as a substrate. The apparent K_m and K_{cat}, in assays with atmospheric equilibration to supply oxygen, were 1.7 μM and 10.5 min^{-1}, respectively. The enzyme is also sensitive to inhibition by acifluorfen with 50% inhibition occurring at concentrations of approximately 4 μM (Fig. 5).

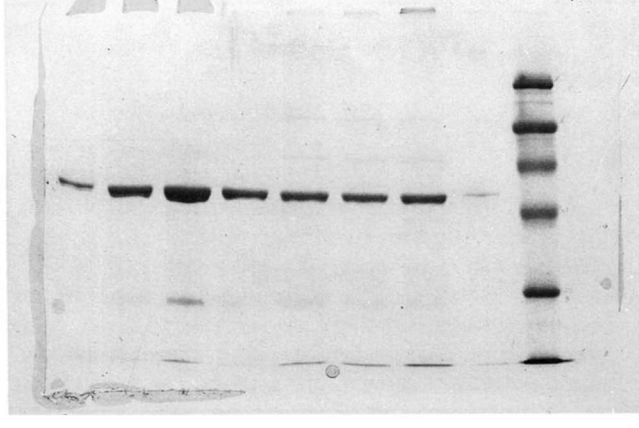

Fig. 3. SDS-PAGE of purified human protoporphyrinogen oxidase. Eight serial fractions from the elution of the Ni^{+2} NTA column are shown. Two-microliter samples of each fraction were loaded onto the gel. The far right hand lane has molecular weight markers myosin, phosphorylase (b), bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor.

Sequence comparisons of human protoporphyrinogen oxidase

Currently, there are now four confirmed oxygen-dependent protoporphyrinogen oxidase sequences. These four from *Bacillus subtilis* (Dailey et al., 1994; Hansson & Hederstedt, 1994), *Myxococcus xanthus* (H.A. Dailey & T.A. Dailey, in prep.), mouse (Dailey et al., 1995), and human (Dailey & Dailey, 1995; Nishimura et al., 1995) are shown in Figure 6 to demonstrate the sequence homologies and identities. Among the four sequences, there is only 15% identity, with the largest single region being near the amino terminus in the putative adenine dinucleotide-binding motif (Wierenga et al., 1986). Allowing for conservative replacements, one finds about 40% homology between the four. A search of the protein database reveals that protoporphyrinogen oxidase shares significant homologies with a number of oxidases that contain an FAD-binding motif at the amino terminus. Typical of this family of enzymes are the monoamine oxidases. A comparison of mammalian protoporphyrinogen oxidases with human monoamine oxidase A and B is shown in Figure 7. There is 16% identity between the four sequences and a little over 30% homology.

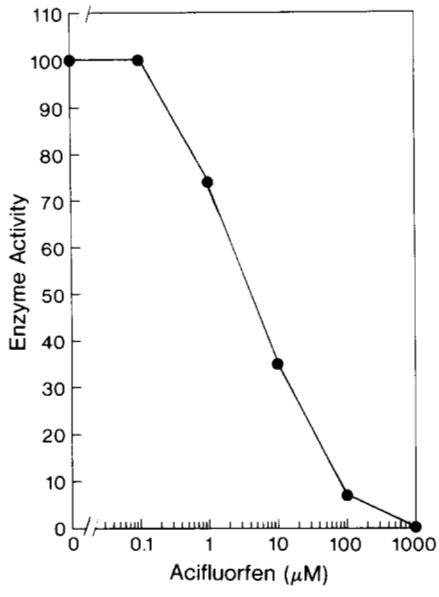

Fig. 5. Acifluorfen inhibition of human protoporphyrinogen oxidase. Enzyme activity is expressed as percent of activity present in the absence of acifluorfen. See text for details.

Discussion

Protoporphyrinogen oxidase has now been cloned from *B. subtilis* (Hansson & Hederstedt, 1992), *M. xanthus* (H.A. Dailey & T.A. Dailey, in prep.), murine erythroleukemia cells (Dailey et al., 1995), and human placenta (Dailey & Dailey, 1995; Nishimura et al., 1995). During the course of the present work, a report appeared describing the cloning and sequence of a human placental enzyme (Nishimura et al., 1995). The published sequence of the cDNA reported for the coding region is identical to what we found, although some slight differences exist in the 3′ and 5′UTR regions. Most significant is that a 61-bp region in the 5′UTR is absent in our clones and that the 5′- and 3′-most sequences are not identical. Although it appears that these end sequence differences may be attributable to their inclusion of vector sequences, it is not possible to be sure because there is no description of their vector construction.

Northern blot analysis of a variety of tissues reveal only a single transcript of 1.8 kb. This is in contrast to mouse, where two transcripts of 1.8 and approximately 3.5 kb were found (Dailey et al., 1996). But, as with the mouse, there is no evidence to suggest tissue specific expression of a particular transcript. Both human and mouse have short 3′UTR regions in the 1.8-kb transcripts and the 5′UTR is approximately 300 bp in length. No predicted stable stem loop structures are found in the short 3′ end, but one large, stable (−70 kcal) 5′UTR stem loop extending from bp 9 to 197 is predicted by GCG (Genetics Computer Group, 1994). In this predicted structure, however, the longest perfect complementary match is only 6 bp, with most being 3 or 4 bp in length. There are no recognizable, previously identified stem-loop structures, such as the iron responsive element (Caughmann et al., 1988), present in either the 5′ or 3′UTR.

The expressed protein has a molecular weight of approximately 51,000 as determined by SDS-PAGE. This is in good agreement with the molecular weight predicted from the derived amino acid sequence. Gel filtration in the presence of 0.2% *n*-octyl-β-D-glucopyranoside yielded a molecular size of 100,000, thus suggesting that the protein exists as a homodimer. The derived amino acid sequence contains a readily identifiable dinucleotide binding motif (Wierenga et al., 1986) at the amino terminal end of the protein that is similar to what was found with the previously cloned and sequenced protoporphyrinogen oxidases from the two bacteria and mouse. The fact that the protein contains an FAD is supported by the ultraviolet (UV)/visible spectrum of the purified protein, which contains the spectra characteristic of oxidized FAD. Interestingly, as the enzyme is purified, there is only 0.5 FAD per protoporphyrinogen oxidase monomer as determined by UV/visible spectra. The addition of FAD to the purified enzyme during in vitro assay does not stimulate activity, and no redox reactive metals were found associated with the protein. The apparent K_m for protoporphyrinogen for the recombinant enzyme is similar to that previously reported for the purified mouse enzyme. The K_{cat}, at about 10 min^{-1} is slow, but within the range of other heme biosynthetic pathway enzymes such as ferrochelatase (Dailey, 1990).

Because the oxidation of protoporphyrinogen to protoporphyrin involves a six-electron oxidation and assuming that the products of the reaction are porphyrin and H_2O_2 as suggested previously (Ferreira & Dailey, 1988), then the reaction scheme would involve three, two-electron reduction/oxidation steps. This would imply that either enzyme-bound or free dihydro- and tetrahydro-porphyrins would exist as reaction intermediates. Resolution of this question should now be possible with the amounts of protein that can be produced.

The amino acid sequence of human protoporphyrinogen oxidase does not contain either a classical membrane-targeting amino-terminal presequence or a membrane-spanning sequence (Milpetz et al., 1995). This is similar to both the mouse and bacterial enzymes. The protein has a calculated isoelectric point of 8.0 and has a relatively unremarkable amino acid composition. One interesting feature that becomes apparent upon comparison of the four reported protoporphyrinogen oxidase-derived amino acid sequences is that there are few conserved charged (Glu + Arg + Lys + Asp = 8) or aromatic residues (3). Indeed the most highly conserved residues among the four sequences are glycine (20) and leucine (12). Although the protein is a dimer and possesses a significant number of conserved leucine residues, the spacing and associated secondary structure predictions do not indicate the presence of a leucine zipper motif.

A survey of the current protein database reveals that there are significant similarities between protoporphyrinogen oxidase and monoamine oxidases. For the mammalian forms of these proteins, these similarities include the following: all have highly homologous regions at the amino terminus that have been identified as the dinucleotide-binding region for the FAD cofactor, all are homodimers of similar sizes, all are localized to membrane surfaces in the intramembrane space of the mitochondrion, none have a typical mitochondrial amino terminal signal peptide, and all catalyze a molecular oxygen-dependent reaction. One difference is that the monoamine oxidases are reported to have one covalently bound FAD (Yasunobu & Tan, 1985), whereas protoporphyrinogen oxidases lack the covalent attachment. This is not unexpected because the Cys residue identified as the linkage group in monoamine oxidases is lacking in protoporphyrinogen oxidase.

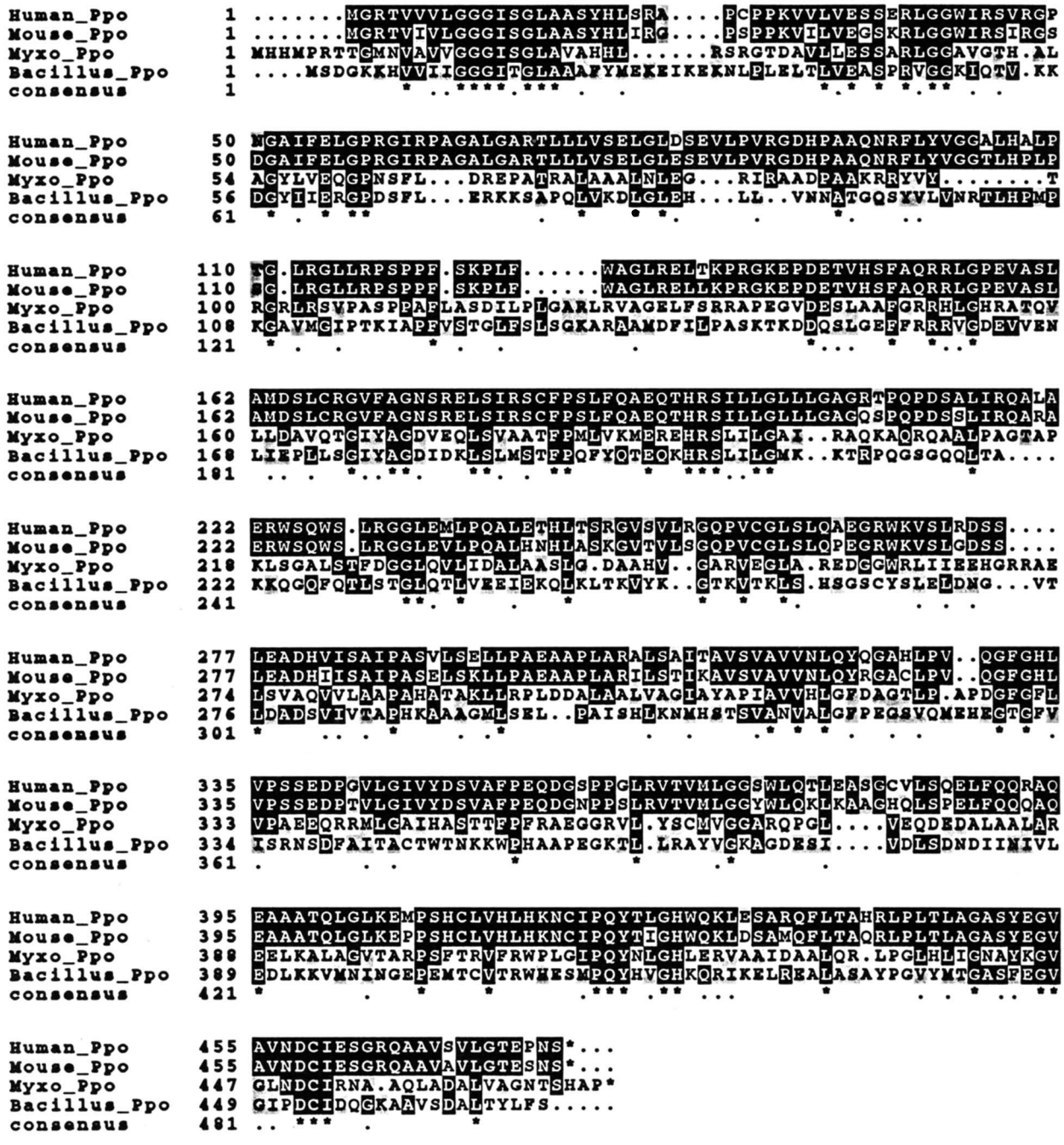

Fig. 6. Sequence comparison of four verified oxygen-dependent protoporphyrinogen oxidases. Genbank accession numbers for the nucleotide sequences are: *Bacillus subtilis*, M97208; *Myxococcus xanthus*, L27429; mouse, U25114; human, U26446. This lineup was generated by the Genetic Computer Group (GCG) program Pileup.

A comparison of the mouse and human protoporphyrinogen oxidases with human A and B monoamine oxidases (Fig. 7) using the alignment program Pileup from GCG results in approximately 17% identity among all four sequences (not counting the carboxyl terminal extension of the monoamine oxidases). Allowing for conservative amino acid replacements results in about 30% homology among all four sequences. Although only human monoamine oxidases are shown in the Figure 6, addition of all known mammalian monoamine oxidase sequences (rat B and bovine A) does not change the comparison significantly because the pictured regions of identity for monoamine oxidases are highly conserved. It should be noted that, among the four currently known sequences for protoporphyrinogen oxidase (human, mouse, *M. xanthus*, and *B. subtilis*), there is only 15% identity and about 40% homology (Fig. 6).

The degree of similarity between protoporphyrinogen oxidases and monoamine oxidases brings into question the evolutionary relationship between these proteins. In considering this topic,

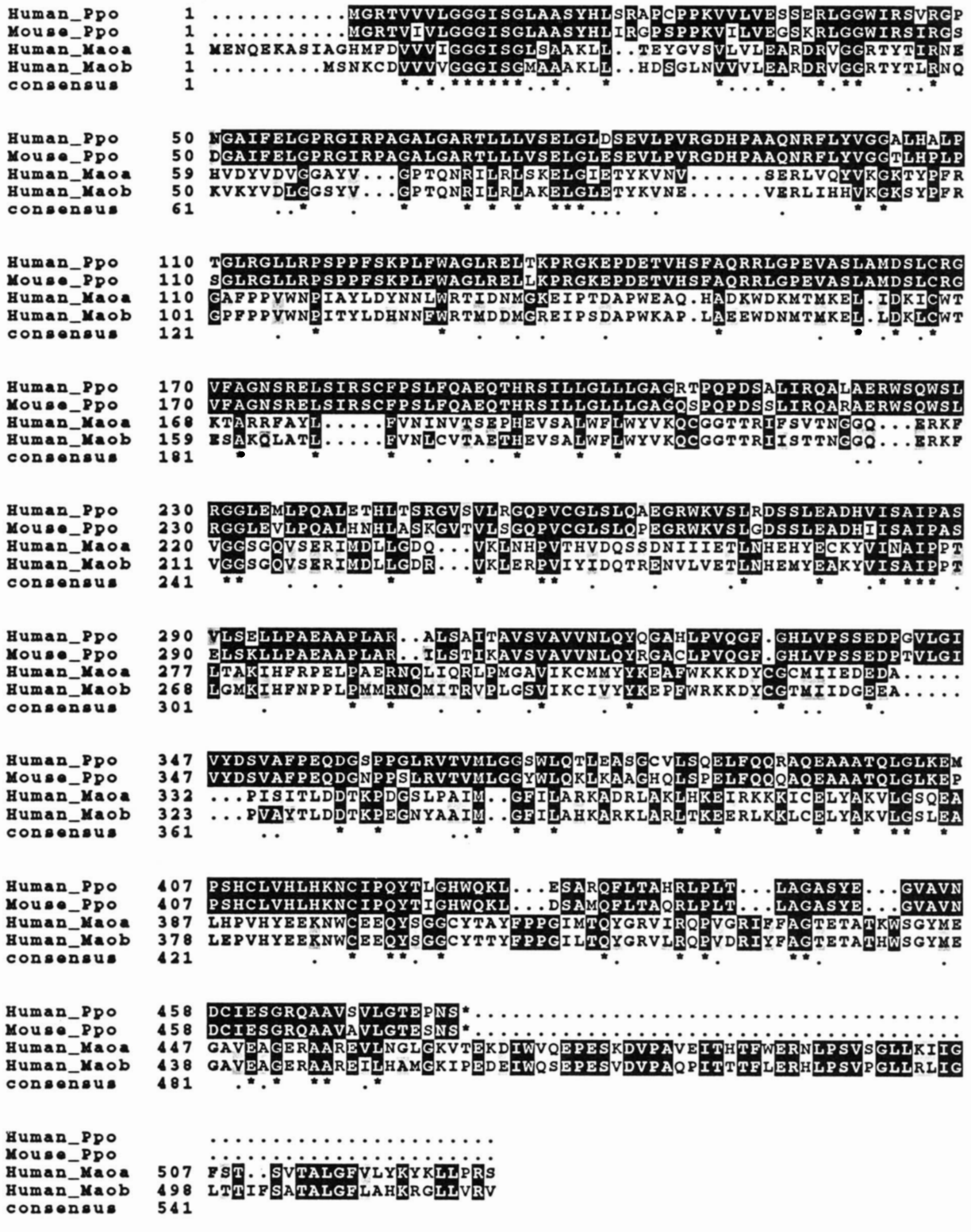

Fig. 7. Amino acid sequence comparison of human and mouse protoporphyrinogen oxidases with human monoamine oxidase (MAO) A and B. The SwissProt accession numbers for MAO A and B are P21397 and P27338, respectively.

it is significant to note that in bacteria at least two distinct protein systems have been identified that carry out oxidation of protoporphyrinogen. The anaerobic bacterium *Desulfovibrio gigas* (Klemm & Barton, 1987, 1989), the facultative bacterium *E. coli* (Jacobs & Jacobs, 1976), and the facultative photosynthetic bacterium *Rhodobacter sphaeroides* (Jacobs & Jacobs, 1981) all appear to possess a respiratory chain-linked multiprotein complex that is responsible for the oxidation. The strict aerobes *B. sub-*

tilis and *M. xanthus*, however, possess an oxygen-dependent, FAD-containing oxidase that is homologous to the mammalian protein described above. It appears that the evolutionary switch from one type of protoporphyrinogen oxidase enzyme to another may correspond to the switch from anaerobic to strictly aerobic growth. Because monoamine oxidases also require molecular oxygen, they would not be expected to be found in anaerobic organisms, but they are found in facultative organisms such as *Klebsiella aerogenes* (Sugino et al., 1991). This may suggest that the primordial molecular oxygen-dependent, FAD-containing oxidase, which gave rise to enzymes such as protoporphyrinogen oxidase, monoamine oxidase, tyramine monooxygenase, and putrescine oxidase, was not evolved initially for tetrapyrrole biosynthesis. Indeed, it would appear from the variety of enzymes in this family, that the original gene may have undergone particularly rapid mutagenesis in order to spawn so many different enzymes so rapidly.

With the cloning and high level expression of human protoporphyrinogen oxidase it now becomes possible to examine naturally occurring mutations in this enzyme that result in the disease variegate porphyria. Due to the chromophoric nature of the cofactor and enzyme products, it should be possible to determine with some precision the underlying defect in catalytic mechanism for each identified missense mutation. These mutations, along with site-directed mutagenesis of selected residues, may aid in both defining catalytic features as well as determining the basis for inhibition of this enzyme by the diphenyl-ether herbicides.

Materials and methods

Library construction

Human placental mRNA was obtained from Clontech. The Zap cDNA Synthesis Kit (Stratagene) was used to convert 4.5 μg of mRNA into lambda phage library. The titer obtained in the primary library was approximately 1×10^7 pfu/mL. This library was excised in vivo to create a plasmid library in pBluescript as described in the manufacturer's protocol. The library was plated onto 20 150-mm^2 LB (Luria Bertani) plates with ampicillin (100 μg/mL) and incubated overnight at 37 °C. Approximately 3×10^5 clones were obtained. Each of the plates was washed with 10 mL of LB, the washes were pooled, and the plasmid DNA was purified from the cells using a Wizard Maxiprep (Promega).

Complementation and sequencing

E. coli strain SASX38 cells, a protoporphyrinogen oxidase-deficient strain (Sasarman et al., 1993), were electroporated with 1 μg each of isolated plasmid DNA in eight separate reactions, outgrown for 1 h at 37 °C with shaking, plated onto LBAmp plates, and incubated overnight at 37 °C. All colonies obtained were screened by polymerase chain reaction (PCR) using pUC universal primers. The complete cDNA sequence, including the 5′ untranslated region (UTR) and 3′ UTR from one clone, was determined by National Biosciences, Inc.

Northern analysis

A probe containing the entire open reading frame of human protoporphyrinogen oxidase cDNA was labeled with digoxigenin by PCR according to a protocol supplied by the manufacturer (Boehringer Mannheim). This probe was hybridized to a human multiple tissue Northern blot (Clontech) according to the manufacturer's instructions.

Plasmid construction

The cDNA coding for protoporphyrinogen oxidase was obtained by using PCR with the clone from the placental library as a template. The 5′ end primer was designed to contain an *Nhe* I site, and the 3′ primer a *Hind* III site. Specifically, the primers used were:

NheI
Sense 5′GC·GCT·AGC·ATG·GGC·CGG·ACC·GTG 3′
Antisense 5′CT·AAGCTT·TCA·GCT·GTT·AGG·TTC·TGT·GCC·CA3′
HindIII

The resulting fragment was cloned into the T vector (Promega) to facilitate restriction digestion, then excised, and cloned into pTrcHis B (Invitrogen) digested with *Nhe* I/*Hind* III. Because it was found that this expressed protein bound poorly to the Ni^{+2} chelate column, a spacer was inserted into the *Nhe* I site to move the His_6 tag away from the FAD binding site. The spacer consisted of a self-complementary oligo, 5′CT·AGT·CCC·AGA·TCT·GGG·A3′, which contained a unique *Bgl* II site to allow for screening. The resulting plasmid was named pHPPO-X.

Expression and purification

E. coli JM109 cells containing pHPPO-X were inoculated into 1 L of Circlegrow media (BIO101) with ampicillin and incubated at 37 °C, with 250 rpm shaking overnight. Cells were harvested by centrifugation (10,000 × *g*, 10 min, 4 °C), the pellet collected and suspended in 60 mL of 50 mM Na phosphate, pH 7.4, 300 mM NaCl, 1.0% *n*-octyl-β-D-glucopyranoside, followed by sonication twice for 30 s. This was then centrifuged (100,000 × *g*, 60 min, 4 °C) to separate the solubilized enzyme from the remaining membrane fraction.

To purify the enzyme, a 3-mL bed volume Qiagen Ni-NTA agarose column was prepared and equilibrated with 50 mM Na phosphate, pH 7.4, 300 mM NaCl, 0.2% *n*-octyl-β-D-glucopyranoside. The solubilized fraction was passed through this column before the column was washed with 50 mL of the equilibration buffer with 25 mM imidazole. Protoporphyrinogen oxidase was eluted with equilibration buffer containing 150 mM imidazole.

Procedures

Protoporphyrinogen oxidase was assayed in sonicated cell extracts using the fluorescence assay described previously (Brenner & Bloomer, 1980b; Dailey & Karr, 1987). Substrate protoporphyrinogen was prepared freshly from protoporphyrin (Porphyrin Products, Logan, Utah) using sodium amalgam reduction. In assays where the herbicide acifluorfen (Chem Services, Westchester, Pennsylvania) was included, a 10-μM or 100-mM stock solution in DMSO was used so that the 1-mL enzyme assay reaction mixture contained less than 1% DMSO.

SDS-PAGE was done using Mini-Protean II Ready Gels (10%) (BioRad, Hercules California) following the manufacturer's instructions, where SDS and 2-mercaptoethanol are added to the sample and running buffer. Gel were stained with Coomassie Blue stain. Visible/UV spectra were recorded with a Varian 219 spectrophotometer. Metal analysis by plasma emission was carried out by the Chemical Analyses Laboratory at the University of Georgia.

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