Identification of an FAD Superfamily Containing Protoporphyrinogen Oxidases, Monoamine Oxidases, and Phytoene Desaturase

EXPRESSION AND CHARACTERIZATION OF PHYTOENE DESATURASE OF MYXOCOCCUS XANTHUS*

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A large number of FAD-containing proteins have previously been shown to contain a signature sequence that is referred to as the dinucleotide binding motif. Protoporphyrinogen oxidase (PPO), the penultimate enzyme of the heme biosynthetic pathway, is an FAD-containing protein that catalyzes the six electron oxidation of protoporphyrinogen IX. Sequence analysis demonstrates the presence of the dinucleotide binding motif at the amino-terminal end of the protein. Analysis of the current data base reveals that PPO has significant sequence similarities to mammalian monoamine oxidases (MAO) A and B, as well as to bacterial and plant phytoene desaturases (PHD). Previously MAOs have been shown to contain FAD, but there are no publications demonstrating the presence of FAD in purified PHDs. We have carried out the expression and purification of PHD from the bacterium Myxococcus xanthus and demonstrate the presence of noncovalently bound FAD. Sequence analysis demonstrate that PPO is closely related to bacterial PHDs and more distantly to plant PHDs and animal MAOs. Interestingly bacterial MAOs are no more closely related to PPOs, PHDs, and animal MAO’s than they are to the unrelated Pseudomonas phenyl hydroxylase. All of the related sequences contain not only the basic putative dinucleotide binding motif that is found frequently for FAD-binding proteins, but they also have high similarity in an approximately 60-residue long region that extends beyond the dinucleotide motif. This region is not found among any other proteins in the current data base and, therefore, we propose that this region is a signature motif for a superfamilly of FAD-containing enzymes that is comprised of PPOs, animal MAOs, and PHDs.

The ability to synthesize tetrapyrroles is one that is found almost universally in living organisms. One of the most abundant of the biological tetrapyrroles synthesized by Bacteria and Eucaryta is protoporphyrin IX or one of its derivatives. Within organisms that possess the ability to synthesize protoporphyrin there appear to exist two mechanisms to catalyze the six electron oxidation of protoporphyrinogen to protoporphyrin. For facultative or anaerobic bacteria there exists a multi-enzyme complex that is linked to the cell’s respiratory chain (1-4), whereas aerobic organisms possess a single protein that utilizes molecular oxygen as terminal electron acceptor (5-8). Currently both systems are named protoporphyrinogen oxidase (PPO) although it is now clear that the biochemical properties of the proteins and the cofactor involvement are distinct.

The oxygen-dependent PPO (EC 1.3.3.4) has been cloned, sequenced, expressed, and purified from both bacterial and mammalian sources (6-12), and it has been demonstrated that this protein is an FAD-containing homodimer with a subunit molecular weight of approximately 50,000. This enzyme is of considerable interest for several reasons. In plants this protein is the target of a large class of commercial herbicides (13, 14), and in man a genetic deficiency of the enzyme results in the disorder variegate porphyria (12, 15-18). In addition, PPO is interesting from a biochemical standpoint since it is an enzyme with only a single FAD, and yet it catalyzes a six electron oxidation of protoporphyrinogen IX to protoporphyrin IX (11).

As one approach to learn more about this enzyme data base searches of Swiss Prot release 32, TIGR, and GenBankTM were carried out to determine if PPO was unique or if it is similar to another class of enzymes that are better characterized structurally. The analysis presented below demonstrates that there exists significant sequence similarities between PPO, monoamine oxidases (MAO), and phytoene desaturases (PHD). Although data have previously been presented demonstrating that PPO (7, 10-12) and MAO (see Ref. 19) contain FAD, no such information was available for a PHD. Herein we report the expression, purification, and demonstration that the bacterial PHD from Myxococcus xanthus contains an FAD. The finding of similarity between PPOs and PHDs is of interest since these two enzymes are the known targets of photoactive herbicides. Data presented also suggest that PPO, MAO, and PHD are members of a superfamilly of FAD-containing proteins that possess a unique fingerprint motif spanning approximately 60 amino acid residues.

MATERIALS AND METHODS

Expression Plasmid Construction—The plasmid pMAR140, containing the coding region for M. xanthus phytoene desaturase, was a gift of Dr. L. J. Shimkets. Primers were designed to carry out the polymerase chain reaction of the gene introducing convenient restriction sites for cloning into the histidine-tagged expression vector pTrexHisC (Invitrogen). Specifically, the sense primer was 5’- GGTAGGCTAGCAGTG-CATCGACACAGGGCAGG-3’; where an NheI site, shown in bold, was

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1 The abbreviations used are: PPO, protoporphyrinogen oxidase; MAO, monoamine oxidase; PHD, phytoene desaturases.
introduced for cloning purposes. The coding region of the phytoene desaturase gene, corresponding to the first 7 amino acids after the 1st methionine in the sequence, are underlined. The antisense primer was 5′-GAACTAGGCTGTCAGCGGCCACCCCTGAC-3′, in which a HindIII site, shown in bold, was introduced for cloning purposes. The 3′-end 30 bp including the TGA stop codon, is underlined. The polymerase chain reaction conditions were as follows: 3 min 95° (1 min 95°, 1 min 62°, 2 min 72°) × 30, 7 min 72°, using 100 μM of each primer and Taq polymerase (Fisher Biotech). The resulting polymerase chain reaction product was cloned into the pGEMT vector (Promega) to facilitate cleavage by restriction enzymes, and the resulting plasmid digested with NheI and HindIII to release the phytoene desaturase DNA. The DNA fragment was then cloned into the NeoHindIII site of pTrHisC. The resulting plasmid was named pPDH.

Expression of PDH—A 1-liter culture of Escherichia coli J1109 containing pPDH was grown at 37 °C overnight with shaking in Circlegrow (Bio101, Inc) supplemented with 100 μg/ml ampicillin. The cells were harvested and resuspended in 60 ml resuspension buffer (50 mM sodium phosphate, pH 5.0, 0.2% wt/v o-phytoglucoside). Additional β-phytoglucoside was added to bring the final concentration to 1.0%, and 10 μg/ml phenylmethylsulfonyl fluoride was added. The cells were sonicated 40 s on high power at 4 °C, and then centrifuged 100,000 χ g at 4 °C for 30 min. The resulting supernatant was loaded onto a Talon (CLONTECH) column that was formed from 3.5 ml of a 50% slurry that had been equilibrated with resuspension buffer. The column was then washed with 20 ml of resuspension buffer, 20 ml of resuspension buffer containing 300 mM NaCl, and then 30 ml of resuspension buffer containing 20 mM imidazole. The purified protein was eluted in resuspension buffer containing 300 mM imidazole, and 0.5 ml fractions were collected.

Data Base Analysis—An initial search of the Swiss Prot data base was done using BLASTP and Wordssearch (20) with the sequence of human protoporphyrinogen oxidase (7) as a probe. Elimination of all sequences lacking an obvious putative dinucleotide binding motif (21) yielded 54 unique protein sequences. Sequence comparisons were carried out by using GCG PileUp (20) using the program’s default values for gap creation and extension penalties. From this initial list those sequences with an amino-terminal dinucleotide binding motif, such as PPO possesses, were selected for further study. These 32 sequences along with the PPO sequence for Deinococcus radiodurans (From TIGR) were submitted to a second PileUp before being phylogenetically sorted using GCG Growtree. All known PPO sequences were included in this analysis. Phenol hydroxylase of Pseudomonas putida was included in this analysis as an outlying, nonrelated sequence to root the trees. This hydroxylase is an FAD-containing protein, but it lacks the dinucleotide binding motif that the other proteins in this analysis possess. Manipulations of sequence length (i.e. truncation of amino or carboxyl-terminal segments to obtain similar protein sizes) had no significant effect on the overall clustering, but for the analysis displayed, the amino-terminal ends (upstream from the dinucleotide binding motif) were truncated from the bacterial MAO sequences. Both Jukes-Cantor and Kimura distance corrections were employed and Growtrees for both neighbor-joining and unweighted pairs group method using arithmetic averages were created.

RESULTS AND DISCUSSION

After the cloning, expression, and purification of PPO (6, 7, 9–12) an analysis of the current data base was performed to identify any related proteins. The analysis yielded a number of interesting results. First of these was the similarity of PPO to both MAO A and MAO B, and PHD. Previously we had reported that PPO had physical properties similar to mammalian MAOs, a close association between PPOs and MAOs was anticipated (7, 11). Among the similarities are that both proteins are nuclear encoded, contain FAD as a cofactor, have a dinucleotide binding motif near the amino terminus, are homodimers with similar subunit molecular sizes, and are mitochondrial membrane-associated proteins that lack a typical amino-terminal targeting sequence. Two major differences between the enzymes are that MAOs catalyze a single two-electron redox step and contain a covalently bound FAD (see Ref. 19), whereas the overall reaction catalyzed by protoporphyrinogen oxidase is a six-electron oxidation (Scheme I) and the protein contains a noncovalently bound FAD (see Ref. 11).

Protoporphyrinogen oxidase

\[
\text{protoporphyrinogen} + 3\mathrm{O}_2 \rightarrow [\text{tetrahydroporphorpinin} \rightarrow \text{dihydroporphorpinin}] \rightarrow \text{protoporphyrin} + 3\mathrm{H}_2\mathrm{O}_2
\]

Phytoene desaturase

\[
\text{phytoene} \rightarrow [\text{phytofluene} \rightarrow \text{tetrahydrocopenence}] \rightarrow \text{neurosperone}
\]

Monoamine oxidase

\[
\text{R-CH}_2-\mathrm{NH}_2 + \mathrm{H}_2\mathrm{O} + \mathrm{O}_2 \rightarrow \text{R-CH} + \mathrm{H}_2\mathrm{O}_2 + \mathrm{NH}_3
\]

Scheme I

To facilitate presentation of over 30 protein sequences and to determine the amount of relatedness of other proteins, a Growtree analysis was performed. As described above, four different analyses were performed: two corrected neighbor-joining, and two corrected unweighted pairs group method using arithmetic averages. Although some variations in relative distances existed among the four trees, all produced the same clustering pattern. The data revealed that two distinct classes of enzymes have structural homology to PPO: animal MAO and PHD (dehydrogenases) (Fig. 1). The pattern places all PPOs in a cluster most closely linked to a cluster of bacterial PHD and more distantly related to mammalian MAO (A and B forms) and plant PHD.

The finding of a close linkage between PPO and PHDs was not anticipated since published reports on PHDs had not demonstrated the presence of FAD. The genes for PHD have been cloned and sequenced from a number of plants and bacteria (see Refs. 22 and 23). The plant PHDs (referred to as pds type PHDs) catalyze the oxidation of phytoene to ω-carotene, a four-electron oxidation that may utilize molecular oxygen, whereas bacterial PHDs (referred to as CrtI type PHDs) convert phytoene all the way to lycopene (an eight-electron oxidation), or to neurosporene (a six-electron oxidation) in reactions that are not stimulated by molecular oxygen (see Ref. 23). The protein from the bacteria Rhodobacter sphaeroides (24) and R. capsulatus (25), which both form neurosporene as product, have been cloned and expressed. The expressed R. capsulatus PHD has been purified to near homogeneity although purification yields were quite low (25). These enzymes are membrane associated and difficult to purify and assay. Because of these factors there are no definitive studies on the enzyme mechanism. Enzymatic studies on the CrtI protein of R. sphoides and R. capsulatus do, however, demonstrate that the enzyme catalyzes three depletions of phytoene to yield neurosporene (Scheme I). In this regard it is similar to PPO, which also carries out a six-electron oxidation. Purified PHD of R. capsulatus was shown to be stimulated by FAD but not NADP(+) (25). Because of the predicted sequence similarity of PHDs to PPOs and MAOs, and the lack of information on PHD, we chose to express and characterize a bacterial PHD from Myxococcus xanthus, which catalyzes a reaction similar to the R. capsulatus enzyme, to determine if its physical properties are similar to those of PPOs and MAOs.

The expression of M. xanthus PHD as a histidine-tagged protein allowed for rapid purification of the protein when we used a procedure that our laboratory has worked out to purify PPOs. The molecular weight of purified PHD is 60,000 as determined by SDS gel electrophoresis (Fig. 2), which is in agreement with the predicted size of 58,600. The calculated extinction coefficient for the apoprotein is 57,000M⁻¹cm⁻¹ at 278 nm and the pI is 9.7. The protein as purified contains nonco-
valently bound 0.5 flavin/monomer of PHD (Fig. 3), and this was identified as FAD by the pH-dependent fluorescence (26). No redox active metals were found associated with PHD. As isolated from the expression system in *E. coli*, PHD contains a small and variable amount of porphyrin. This amount of bound porphyrin represents a few percent of the total PHD present, but because of the presence of porphyrin and the similarities with PPO, PHD was assayed for PPO activity. PHD was found to have no significant PPO activity, and the PHD expression vector did not complement *E. coli* SAS38X cells, which lack PPO activity (27) (data not shown).

Based upon protein size, amino acid sequence homology (including the presence of the highly conserved amino-terminal dinucleotide binding motif), the presence of FAD as a cofactor, and the similarity in the reactions catalyzed, it would appear that phytoene desaturase might catalyze an oxygen-dependent oxidation of phytoene. However, the observations that carotene biosynthesis in photosynthetic bacteria is maximal during anaerobic photosynthetic growth (28) and that mRNA levels for this enzyme in *R. sphaeroides* are decreased under aerobic conditions (24) but are unchanged under the same conditions in *R. capsulata* (29) do raise questions that may be addressed experimentally now that the protein can be expressed and purified.

As shown in the Growtree analysis (Fig. 1), mammalian MAOs cluster together as would be expected, but, interestingly, the bacterial MAOs are quite distinct phylogenetically. In fact the bacterial MAOs appear no more closely linked to animal MAOs than to phenol hydroxylase. One point of interest is the presence of an unidentified yeast open reading frame (P32614), which is a yeast unidentified open reading frame. The analysis shown is for Kimura corrected unweighted pairs group method using arithmetic averages.

![Fig. 1. Growtree analysis. The analysis shown is for all published sequences of protoporphyrinogen oxidases (Ppo), monoamine oxidases (Mao a, b, c), phytoene desaturase (Phytdeh), hydroxyneurospermine desaturase (Hnde), methoxyneurospermine desaturase (Mnde) and phenol hydroxylase (phen_Hydrox). Species names are clearly identified except for P32614, which is a yeast unidentified open reading frame. The analysis shown is for Kimura corrected unweighted pairs group method using arithmetic averages.](image1)

![Fig. 2. SDS gel electrophoresis of purified M. xanthus phytoene desaturase. The gel photograph shows molecular weight markers on the left side, solubilized cell extract, and purified histidine-tagged phytoene desaturase (10 μg).](image2)

![Fig. 3. UV/visible spectrum of purified M. xanthus phytoene desaturase and acid-extracted flavin. The upper figure shows the spectrum of 20 μM purified enzyme. The inset is an enlargement of the visible spectrum. The lower figure is the spectrum of 5% trichloroacetic acid extracted flavin from the purified enzyme. The concentration of the extracted flavin fraction is one-half of that shown for the enzyme in the upper figure.](image3)
been reported that these enzymes are biochemically distinct from the microbial phytoene desaturases (see Refs. 23 and 30). Whereas it is clear from sequence analysis that these two groups of enzymes are distinct, it appears that they all are members of a protein superfamily.

Examination of the putative dinucleotide binding motif sequences (Fig. 4) clearly shows that significant identity and close homologies exist among all of the enzymes and that this identity extends past the requisite Glu (or Asp) (21). When a Growtree analysis of this approximately 60-residue long segment is carried out using the same parameters described above the cluster pattern found in the tree is very similar to that obtained when one uses the complete protein sequences (Fig. 1). The only significant change is that the sequence for Myxococcus hydroxyneurosporine dehydrogenase becomes clustered with protoporphyrinogen oxidase sequences. Such findings suggest that members of this superfamily have arisen via divergence from a common ancestor and did not acquire their exact mode of action is unknown. It has been suggested that diphenyl ether herbicides may be substrate analogs for PPO but lack of purified PHD preparations has slowed research on these enzymes. Whereas it is clear from sequence analysis that these two enzymes are both sites of action of plant herbicides. These classes of herbicides comprise two of the largest groups of currently utilized plant herbicides, but at the present time their exact mode of action is unknown. It has been suggested that diphenyl ether herbicides may be substrate analogs for PPO but lack of purified PHD preparations has slowed research on these enzymes.

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FIG. 4. Pileup alignment of the proposed superfamily fingerprint motif. The dinucleotide binding motif of Wierenga et al. (21) is underlined, and the conserved glutamate is marked (5). The sequence of the proposed fingerprint for this superfamily is: UG(G/A)G(U/A)G(Y/W)X6–12(G/V)(G/A)G(Y/W)X3(G/D/E)(L/V)X2(L/V)X2(A/S)(D/E)(L/V)X3(U/E)X4(U/E)X4(G/G)G where U is a hydrophobic residue and X is any residue. Note the almost complete identity in this region for all plant phytoene desaturases. Abbreviations are as in the legend to Fig. 1 except that Dbm stands for dinucleotide binding motif.
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