Protoporphyrinogen oxidase (EC 1.3.3.4) catalyzes the six electron oxidation of protoporphyrinogen IX to protoporphyrin IX. The enzyme from the bacterium Myxococcus xanthus has been cloned, expressed, purified, and characterized. The protein has been expressed in Escherichia coli using a Tac promoter-driven expression plasmid and purified to apparent homogeneity in a rapid procedure that yields approximately 10 mg of purified protein per liter of culture. Based upon the deduced amino acid sequence, the molecular weight of a single subunit is 49,387. Gel permeation chromatography in the presence of 0.2% n-octyl-β-D-glucopyranoside yields a molecular weight of approximately 100,000 while SDS gel electrophoresis shows a single band at 50,000. The native enzyme is, thus, a homodimer. The purified protein contains a non-covalently bound FAD but no detectable redox active metal. The M. xanthus enzyme utilizes protoporphyrinogen IX, but not coproporphyrinogen III, as substrate and produces 3 mol of H2O2/mol of protoporphyrin. The apparent Km and Kcat for protoporphyrinogen in assays under atmospheric concentrations of oxygen are 1.6 μM and 5.2 min⁻¹, respectively. The diphenyl ether herbicide acifluorfen at 1 μM strongly inhibits the enzyme’s activity.

The penultimate step in the heme biosynthetic pathway, the six electron oxidation of protoporphyrinogen IX to protoporphyrin IX, is catalyzed by the enzyme protoporphyrinogen oxidase (EC 1.3.3.4) (1-3). In eukaryotes this enzyme is located on the cytosolic side of the inner mitochondrial membrane and utilizes molecular oxygen as its terminal electron acceptor. The enzyme from two prokaryotes have been cloned, sequenced, expressed, and partially characterized. The enzyme from Bacillus subtilis (4, 5) is similar to the eukaryotic enzyme in that it contains a flavin and utilizes molecular oxygen as terminal electron acceptor. However, its substrate specificity is much broader than the eukaryotic enzyme since it will oxidize not only protoporphyrinogen IX, but also the pathway intermediate coproporphyrinogen III. In addition it is resistant to inhibition by the herbicide acifluorfen which strongly inhibits the eukaryotic enzyme. Protoporphyrinogen oxidase has also been cloned and expressed from Escherichia coli (6). The cloned protein is smaller in size than the B. subtilis and eukaryotic enzymes, does not contain an FAD binding motif, and is obligatorily coupled to the cell’s respiratory chain. Based upon previous data from Desulfovibrio gigas (7) it would appear that the cloned E. coli enzyme is a subunit of a multi-protein complex. Data base searches using the B. subtilis protoporphyrinogen oxidase derived amino acid sequence yielded two similar bacterial sequences (8). In an effort to expand our knowledge about this enzyme and to discover if the herbicide resistance and broad substrate specificity found with the B. subtilis enzyme are characteristic of the prokaryotic, oxygen dependent enzymes, we expressed, purified, and characterized protoporphyrinogen oxidase of the bacterium Myxococcus xanthus.

MATERIALS AND METHODS

Plasmid Construction—The plasmid pJL S43 (9) was kindly supplied by L. Shimkets. The DNA for the putative protoporphyrinogen oxidase coding region was obtained by using polymerase chain reaction with the oligonucleotide primers: sense, 5'-CCATG-GAC-CAT-CAC-CAT-CAC-CAC-ATG-CCG-AGG-ACA-3'; antisense, 5'-CAAGCTT-CTA-CGG-GGC-CTG-GGA-GGT-3'. The 5' end primer was designed to contain a Ncol restriction site as well as codons for an additional four His residues so that the expressed protein would contain a His-6 tag for Ni-chelate chromatography. The 3' end was extended to create a HindIII site. This fragment was cloned into the Tac-driven expression vector pTF20E which is a derivative of pBTac1 (Boehringer Mannheim) in which an optimally spaced ribosomal binding site, T7 enhancer and ATG start site (10) were placed immediately downstream from the Tac promoter. The expression vector pMx×PPO was transformed into E. coli JM109 for protein expression.

Expression and Purification—JM109 cells containing pMx×PPO were inoculated into 1 liter of Cirldegrow medium (BIO101) with 100 μg/ml ampicillin and incubated at 37 °C with 250 rpm shaking overnight. Cells were harvested by centrifugation (10,000 × g, 10 min, 4 °C), and the pellet was collected and suspended in 60 ml of 50 mM sodium phosphate, pH 7.4. The cells were lysed by sonication and the cell membrane fraction separated from the cytoplasmic fraction by centrifugation at 100,000 × g, 60 min, 4 °C. Enzyme was solubilized from the isolated membrane fraction by resuspension of the pellet in 60 ml of 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 0.5% n-octyl-β-D-glucopyranoside followed by sonication for 15 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 sodium 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 20 mM imidazole. Protoporphyrinogen oxidase was eluted with equilibration buffer containing 150 mM imidazole.

Procedures—Protoporphyrinogen oxidase was assayed as described previously (11, 12). The assay buffer contained 100 mM sodium phosphate, pH 7.4, 0.1 mM EDTA, and 0.1% Tween 20. Porphyrinogen substrate was prepared via reduction with sodium amalgam and was used immediately. In assays where acifluorfen (Chem Service, Inc.) was present the acifluorfen was added from a stock solution in dimethyl sulfoxide alone to the assays had no effect on observed enzyme activity. All assay procedures were carried out in a darkened room.

SDS gel electrophoresis was carried out with Mini-Protean II Ready Gels (Bio-Rad, Hercules, CA). 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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Georgia. Femtomole sequencing (Promega) with 35S-dATP was employed for DNA sequencing. Additional DNA sequencing was also carried out by the Molecular Genetics Facility at the University of Georgia.

Quantitation of H₂O₂ produced was achieved using scopoletin (13). Briefly, assays were set up where complete conversion of protoporphyrinogen to protoporphyrin was achieved within 5 min. Duplicate samples were prepared for porphyrinogen concentrations of 0, 10, 25, and 50 μM. Complete conversion of substrate to product was verified spectrophotometrically before H₂O₂ concentration was determined. A standard curve for scopoletin quantitated H₂O₂ was constructed using a H₂O₂ solution whose concentration had been verified with 4-aminoantipyrine (14).

For flavin determination samples of purified enzyme were treated with 5% (w/v) trichloroacetic acid or 50% acetonitrile. Either of these procedures precipitated the protein and released the flavin into solution. Identification of FAD was made based upon the pH-dependent change in fluorescence (15). For quantitation a molar extinction coefficient of 13,000 M⁻¹ cm⁻¹ at 450 nm was employed. To determine flavin to protein stoichiometry, protein concentration was determined spectrophotometrically using a molar extinction coefficient of 21,700 at 275 nm which is based upon the amino acid composition. All enzyme preparations that were used for flavin determination were subjected to SDS gel electrophoresis to ensure that the particular enzyme preparation was homogeneous by this standard.

RESULTS

cDNA Sequence of M. xanthus Protoporphyrinogen Oxidase—The direct translation of the previously published M. xanthus DNA sequence for an unidentified open reading frame (which we have now shown codes for protoporphyrinogen oxidase) yields a protein whose derived amino acid sequence is smaller than the protein we have expressed and characterized herein. It was suggested by Hansson and Hederstedt (8) that a T insertion at base pair 388 of the published DNA sequence would yield a sequence that had homology to the protoporphyrinogen oxidase of B. subtilis. To confirm and identify any insertion or deletion we sequenced the region of M. xanthus DNA from plasmid pJL S45 that encodes protoporphyrinogen oxidase. It was found that there was not a T insertion at bp 388 as suggested but rather a single C insertion immediately after bp 376 (data not shown). This correction has been entered with GenBank™ (accession no. M73709). The sequence of the correct translation product is shown in Fig. 1 along with the three other known oxygen dependent protoporphyrinogen oxidases.

Expression and Purification—The addition of four His residues after amino acid number 2 to yield six consecutive His residues allowed the use of a Ni-chelate column for purification. For high yields it was found important to keep protease inhibitors present in all buffers and to carry out the chromatography step immediately following cell fractionation. While the expressed enzyme was found membrane associated in E. coli, at high levels of expression it was also found in the cytoplasmic fraction. Thus, the addition of the four his residues at the amino terminus did not appear to affect the cellular location of the protein. The possibility that this extension has an adverse affect on this particular enzyme seems unlikely since the amino terminus of known protoporphyrinogen oxidases (along with monoamine oxidases which are similar to this enzyme) are highly variable in length and amino acid composition in the region upstream from the putative dinucleotide binding motif (C. M. Frazier, GenBank™ accession nos. U39704 and L43967).

FIG. 1. Amino acid sequence of protoporphyrinogen oxidase of M. xanthus. The protein sequence was derived from the previously published nucleotide sequence of Li et al. (9) (GenBank™ M73709). Also shown in this figure are sequences for human (GenBank™ U26446), mouse (GenBank™ U25114), and B. subtilis (GenBank™ M97208) protoporphyrinogen oxidases (Ppo). This alignment was generated by the GCG program Pileup. The underlined region represents the putative dinucleotide binding motif (C. M. Frazier, GenBank™ accession nos. U39704 and L43967).

1 H. A. Dailey and T. A. Dailey, unpublished data.
recovery of only a single protein. The expression and purification described above yields a single protein band on SDS gel electrophoresis (Fig. 2) of estimated molecular weight of 50,000. This corresponds well to the molecular weight of 49,387 based upon predicted amino acid sequence.

Properties of the Purified Enzyme—The visible/ultraviolet spectrum of purified M. xanthus protoporphyrinogen oxidase is shown in Fig. 3. The spectra clearly is consistent with the presence of FAD and the amount of FAD was determined to be 0.4–0.5 FAD per subunit. Growth of the cultures with 1 mM riboflavin added during the final 2 h of induction did not cause any alteration in the ratio of FAD to enzyme, but did increase the amount of enzyme recovered by approximately 4-fold. Five percent trichloroacetic acid precipitates the protein and releases free flavin into solution. Visible spectroscopy shows a typical FAD/FMN spectrum and FAD was identified by its pH-dependent change in fluorescence as described previously (15). In the spectrum of the isolated enzyme there is a small feature at around 410 nm which is attributable to a small amount (less than 0.05 mol/mol enzyme) of residual enzyme product (protoporphyrin). Based upon the derived amino acid sequence, the extinction coefficient at 275 nm is 21,700 M/cm.

Gel filtration on a Sephacel S-300 column (25 × 2 cm) in the presence of 20 mM sodium phosphate, pH 7.4, 0.2% n-octyl-β-D-glucopyranoside resulted in the elution of a single protein peak with an estimated molecular weight of 100,000 (Fig. 4). Metal analysis did not demonstrate the presence of any redox active metal (data not shown).

The M. xanthus enzyme is strongly inhibited by the herbicide acifluorfen (Fig. 5). This level of inhibition is similar to what is seen with the mammalian enzyme and unlike what was reported for the B. subtilis protein. Preliminary kinetic experiments suggest that acifluorfen may be a slow binding competitive inhibitor (data not shown). The M. xanthus enzyme uses protoporphyrinogen IX as substrate with an apparent $K_m$ of 1.6 μM and $V_{max}$ of 5.2 min$^{-1}$ and it generates 3 mol of H$_2$O/1 mol of porphyrinogen (average of six determinations was 3.0 ± 0.3). Coproporphyrinogen III is not a substrate for this enzyme. Addition of FAD to reaction mixtures had no detectable effect upon enzyme activity.

**DISCUSSION**

The enzymatic conversion of protoporphyrinogen IX to protoporphyrin IX was first unequivocally demonstrated by Poulson and Polglase in 1975 (2). While a number of papers have appeared on the characterization of the purified eukaryotic enzyme (12, 16–18), little biophysical or accurate kinetic data were available since the enzyme is present in low amounts in cells and is difficult to purify.

In the current study we have presented data on the expression and characterization of protoporphyrinogen oxidase from the Gram-negative bacterium M. xanthus. Interestingly the DNA sequence for this enzyme was reported in 1992 as part of a study from Shimkets’ group on genes involved in the develop-
The enzyme was incubated in the assay reaction mixture, without porphyrinogen substrate, for 5 min prior to addition of substrate.

The enzyme has a molecular weight of 49,387 as determined from the derived amino acid sequence which is in good agreement with what is found by SDS gel electrophoresis. The pure enzyme in solution containing 0.2% detergent exists as a homodimer with no detectable monomer form. Visible spectra of purified protein shows that it possesses a flavin cofactor as suggested from the sequence which contains a dinucleotide (FAD) binding consensus motif (22). The stoichiometry of the FAD to protein in all preparations obtained to date is only about 0.5 and this may reflect that the flavin readily dissociates, or that the dimer form of the enzyme possesses only one FAD per dimer. While the possibility exists that the cells are unable to synthesize sufficient FAD to provide two FAD per dimer, this seems less likely since addition of 1 μM riboflavin to the bacterial culture during the last 2 h of induction did not have a discernable effect upon the FAD content of the purified enzyme. Similar findings of less than stoichiometric amounts of cofactor have been reported for monoamine oxidase which is also a dimeric FAD containing oxidase although its FAD is covalently bound (23).

Kinetic analysis of M. xanthus protoporphyrinogen oxidase demonstrates that it is more similar to the previously characterized eukaryotic enzymes (16–18) than to the only other characterized prokaryotic enzyme from B. subtilis (5, 8). Unlike the bacillus enzyme, the M. xanthus enzyme does not oxidize coproporphyrinogen III and is strongly inhibited by acifluorfen. These data show that the bacillus enzyme’s properties are not representative of all oxygen dependent prokaryotic protoporphyrinogen oxidases. While it will be necessary to characterize the enzyme from additional bacteria before determining which of these enzymes is most widely distributed among prokaryotes, the available data on B. subtilis ferrochelatase, the terminal heme biosynthetic pathway enzyme, demonstrate that this bacillus enzyme also possesses some properties such as protein solubility and metal specificities that are unique among the currently characterized ferrochelatases (24, 25). These observations suggest that a class of bacteria represented by B. subtilis may have evolved a slightly altered way of dealing with the arrangement and intracellular compartmentation of the terminal segment of the heme biosynthetic pathway. The findings that the M. xanthus protoporphyrinogen oxidase is as sensitive to acifluorfen as the eukaryotic enzymes (26–28) demonstrates that the basis for the B. subtilis enzyme’s resistance to this herbicide must be due to a property unique to bacillus and not a more general structural difference between the prokaryotic and eukaryotic enzymes. Since it has been suggested that acifluorfen is a competitive inhibitor of protoporphyrinogen oxidase because it bears a structural resemblance to one-half of the porphyrinogen macrocycle, it will be of interest to see if acifluorfen resistance and broadened substrate specificity are necessarily coupled.

Comparison of the derived amino acid sequence for protoporphyrinogen oxidase from M. xanthus with B. subtilis (4), mouse (29), and human (20, 30) show that there is only 15% identity among all sequences. Between the two bacterial sequences there is 23% identity. If one considers conservative amino acid differences between two different enzymes to catalyze this step (3–8). Protoporphyrinogen oxidase activity in E. coli has been found to involve at least two distinct gene products (hem G (6) and hem K (19)). Neither of these encoded proteins resemble the FAD containing protoporphyrinogen oxidase of mouse, human and B. subtilis although similar derived amino acid sequences have been found in the Haemophilus influenzae and Mycoplasma genitalium genomes. It now seems clear that among bacteria two distinct protoporphyrinogen-oxidizing systems are found; the FAD-containing, oxygen-dependent homodimer enzyme, and the multisubunit, respiratory chain-linked enzyme system as typified by E. coli and D. gigas (7). Limited data suggest that anaerobes or facultative organisms may possess the multisubunit enzyme that is obligatorily linked to the cell’s respiratory chain (3, 6, 7), whereas strict aerobes possess an oxygen dependent protoporphyrinogen oxidase (3, 5, 8). Of these enzymes only the protein from B. subtilis has been cloned and expressed (5, 8). This enzyme was found to have both sequence and catalytic similarities to the eukaryotic enzymes although it differed significantly in that its substrate specificity was much broader and it was not inhibited by the diphenyl ether herbicide, acifluorfen. Since this second property may be a desirable one to clone into selected crop plants, it was of interest to determine if acifluorfen resistance is a general property of all protoporphyrinogen oxidases.

Previous work has shown that bacteria apparently utilize two of different enzymes to catalyze this step (3–8). Protoporphyrinogen oxidase activity in E. coli has been found to involve at least two distinct gene products (hem G (6) and hem K (19)). Neither of these encoded proteins resemble the FAD containing protoporphyrinogen oxidase of mouse, human and B. subtilis although similar derived amino acid sequences have been found in the Haemophilus influenzae and Mycoplasma genitalium genomes. It now seems clear that among bacteria two distinct protoporphyrinogen-oxidizing systems are found; the FAD-containing, oxygen-dependent homodimer enzyme, and the multisubunit, respiratory chain-linked enzyme system as typified by E. coli and D. gigas (7). Limited data suggest that anaerobes or facultative organisms may possess the multisubunit enzyme that is obligatorily linked to the cell’s respiratory chain (3, 6, 7), whereas strict aerobes possess an oxygen dependent protoporphyrinogen oxidase (3, 5, 8). Of these enzymes only the protein from B. subtilis has been cloned and expressed (5, 8). This enzyme was found to have both sequence and catalytic similarities to the eukaryotic enzymes although it differed significantly in that its substrate specificity was much broader and it was not inhibited by the diphenyl ether herbicide, acifluorfen. Since this second property may be a desirable one to clone into selected crop plants, it was of interest to determine if acifluorfen resistance is a general property of all bacterial oxygen dependent protoporphyrinogen oxidases and, if so, to identify the structural feature that imparts this property.

Data presented above demonstrate that the previously published open reading frame from the myxobacterium M. xanthus codes for the enzyme protoporphyrinogen oxidase. The expressed protein is an oxygen dependent, flavin containing enzyme that is similar to the mammalian enzyme and the enzyme from B. subtilis. The protein which has two amino-terminal his residues was expressed in E. coli using a vector in which four additional his residues were added to create a 6-his tag for purification via Ni-CHelate chromatography. The expressed enzyme is found in both membrane and cytoplasmic fractions, but the purified enzyme rapidly precipitates out of solution in the absence of detergent and purification requires at least 0.2% octyl glucoside. Once purified the protein is stable for weeks at 4°C.

Kinetic analysis of M. xanthus protoporphyrinogen oxidase demonstrates that it is more similar to the previously characterized eukaryotic enzymes (16–18) than to the only other characterized prokaryotic enzyme from B. subtilis (5, 8). Unlike the bacillus enzyme, the M. xanthus enzyme does not oxidize coproporphyrinogen III and is strongly inhibited by acifluorfen. These data show that the bacillus enzyme’s properties are not representative of the only other characterized prokaryotic protoporphyrinogen oxidases. While it will be necessary to characterize the enzyme from additional bacteria before determining which of these enzymes is most widely distributed among prokaryotes, the available data on B. subtilis ferrochelatase, the terminal heme biosynthetic pathway enzyme, demonstrate that this bacillus enzyme also possesses some properties such as protein solubility and metal specificities that are unique among the currently characterized ferrochelatases (24, 25). These observations suggest that a class of bacteria represented by B. subtilis may have evolved a slightly altered way of dealing with the arrangement and intracellular compartmentation of the terminal segment of the heme biosynthetic pathway. The findings that the M. xanthus protoporphyrinogen oxidase is as sensitive to acifluorfen as the eukaryotic enzymes (26–28) demonstrates that the basis for the B. subtilis enzyme’s resistance to this herbicide must be due to a property unique to bacillus and not a more general structural difference between the prokaryotic and eukaryotic enzymes. Since it has been suggested that acifluorfen is a competitive inhibitor of protoporphyrinogen oxidase because it bears a structural resemblance to one-half of the porphyrinogen macrocycle, it will be of interest to see if acifluorfen resistance and broadened substrate specificity are necessarily coupled.

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2 O. White, GenBank™ accession nos. U32799 and L42023.
3 C. M. Frasier, GenBank™ accession nos. U39704 and L43967.
substitutions then there is about 35–40% homology among the four sequences. While the regions of identity appear to be relatively randomly distributed throughout the proteins, there are several discrete regions of homology. The most obvious of these regions is the dinucleotide binding motif (22) found at the amino-terminal end of all four sequences. The structural/functional purposes of the remaining regions are currently unknown, but the lack of an identifiable membrane spanning region in any of the sequences rules out that possibility.

The reaction catalyzed by protoporphyrinogen oxidase is a six-electron oxidation. Previously we have shown that three O₂ are consumed per porphyrinogen substrate (21) and above we document that three H₂O₂ are produced. Studies by others on crude enzyme extracts did not detect the in vitro accumulation of a tetra or dihydro porphyrin intermediate. If the enzyme contains only a single FAD and no additional redox active cofactors or metals, then the reaction must proceed in three distinct steps unless residue side chains such as tyrosine are involved. Among currently published sequences there is only one conserved tyrosine residue. With the ability to produce and purify this enzyme in milligram quantities as well as the possibility to carry out site-directed mutagenesis on the cloned enzyme, we should now be able to determine the sequence of catalytic events in the oxygen dependent conversion of the porphyrinogen to porphyrin.

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