Anaerobic Oxidation of p-Cresol Mediated by a Partially Purified Methylhydroxylase from a Denitrifying Bacterium

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Anoxic cell extracts of a denitrifying bacterial isolate (PC-07) were shown to oxidize p-cresol to p-hydroxybenzoate. Oxidation of the substrate was independent of molecular oxygen and required nitrates as the natural terminal electron acceptor. Two enzyme activities were implicated in the pathway utilized by PC-07.

A p-cresol (pCr)-utilizing, denitrifying bacterium, PC-07, has been previously isolated and described (4). The isolate is one member of a bacterial coculture comprising two denitrifying species which interdependently utilize pCr as the sole source of carbon for growth under anaerobic conditions (5). Initially, substrate oxidation to p-hydroxybenzoate (pOHB) is mediated by the PC-07 isolate and provides the ring fission substrate to the second member of the coculture; neither isolate is able to use the respective substrate of its partner for growth under anaerobic conditions. The PC-07 isolate, however, can utilize pCr as its sole source of carbon for growth under aerobic conditions. Studies with whole-cell suspensions of PC-07 have established the pathway for the anaerobic oxidation of pCr to pOHB via p-hydroxybenzyl alcohol and p-hydroxybenzaldehyde (pPHBZ) intermediates. Substrate oxidation depends on the stoichiometric reduction of NO3⁻ to N₂.

The studies presented here further characterized the anaerobic oxidation of pCr by cell extracts of the PC-07 isolate. In addition, these studies described the partial purification and characterization of a pCr methylhydroxylase enzyme (PCMH) which mediates the initial oxidation of pCr.

MATERIALS AND METHODS

Materials and reagents. Trizma base, phenazine methosulfate (PMS), 2,6-dichloro phenol-indophenol (DCPIP), bovine serum albumin, DNase, and NAD (disodium salt) were from Sigma Chemical Co. (St. Louis, Mo.); DEAE-cellulose (DE-52) was from Whatman, Ltd. (Maidstone, Kent, England); DEAE-Sephacel and SI-17 anion-exchange resin were from Pharmacia Fine Chemicals (Piscataway, N.J.); pCr, m-cresol, o-cresol, p-chlorotoluene, pOHB, p-methylcatechol, 2,4-dimethylphenol (2,4-DMP), and 3,4-dimethylphenol (3,4-DMP) were from Aldrich Chemical Co. (Milwaukee, Wis.); p-hydroxybenzyl alcohol, pHBZ, p-thiocresol, and t-butylated cresol were from Fluka Chemical Co. (Hauppauge, N.Y.). 4-Methylcatechol was purified by sublimation, and all other chemicals were of highest purity commercially available. O2-free argon gas (>99.999%) was obtained from Linde Specialty Gases (Somerset, N.J.).

Growth and harvest of cells. Pure cultures of the pCr-oxidizing, denitrifying isolate, PC-07, described elsewhere (4), were routinely grown in 9-liter batch cultures under strict anaerobic conditions. For growth under denitrifying conditions, a modified Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with KNO₃ (50 mM) and pCr (3 mM) was used (5). A 25-h denitrifying culture of the PC-07 isolate provided a 7% inoculum. Incubation was at 25°C in a butyl rubber-stoppered 12-liter glass carboy flushed with O₂-free argon gas. A unidirectional gas train apparatus allowed excess pressure from N₂ evolved during denitrification to be vented without any atmospheric O₂ contamination. Cells were harvested after 20 h or when at least 50% of the pCr in the medium had been oxidized to pOHB. After Gram staining and a microscopic check for culture purity, the culture broth was dispensed under an argon stream into polypropylene tubes, capped, and centrifuged for 30 min at 10,000 x g. The cell pellets were suspended in anoxic 50 mM phosphate buffer (pH 7.1), pooled, and recentrifuged for 30 min at 10,400 x g.

Preparation of cell extracts. The cell pellet was subjected to three freeze-thaw cycles and suspended in anoxic 40 mM Tris hydrochloride buffer (pH 7.6). A French pressure cell was used to rupture the cells to 16,000 lb/in². After rupture, the pressate was gassed with argon, treated with a small amount of DNase to reduce viscosity, and centrifuged at 13,300 x g for 50 min to remove cell debris. The supernatant served either as the cell extract in the reported experiments or was further purified by anion-exchange chromatography. Although the cell extract of PC-07 was initially prepared under the described anoxic conditions from anaerobically...
grown cells, the sought-after enzyme activities were also present and stable in aerobically prepared extracts of aerobically grown PC-07 cells and exhibited a similar elution profile during protein purification and an identical substrate range and affinity (e.g., \( K_m \)).

**Partial purification of PCMH protein.** Routinely, the cell extract was amended to 10% ethanol and 10% glycerol and loaded at a rate of 1.0 ml/min on a DEAE-Sephaloc (or DE-52) column (10 by 2.6 cm) prequilled with PEG buffer (50 mM phosphate, 10% ethanol, 10% glycerol at pH 7.45). Bound protein was eluted with a 0 to 400 mM KCl gradient in 400 ml of PEG buffer. The collected fractions were assayed for protein at \( A_{280} \), and 150-μl samples were tested for PCMH activity. Owing to the low specific activity of PCMH in the eluted fractions, the DCPIP-PMS assay (described below) was modified to measure endpoint activity after 10 to 20 min of incubation. Those fractions exhibiting PCMH activity were pooled and dialyzed overnight against 40 mM Tris hydrochloride (pH 7.6). The dialysate was concentrated on a second DEAE-Sephaloc column (4.5 by 0.8 cm) prequilled with 50 mM Tris hydrochloride (pH 7.6). KCl (700 mM) in 50 mM Tris hydrochloride was used to elute the protein, which could be clearly seen as a dark tannish red band. Eluted fractions with PCMH activity were dialyzed overnight against 40 mM Tris hydrochloride (pH 7.6) and stored at \(-20^\circ\)C.

**Preparation of PCMH for spectral studies.** Fast protein liquid chromatography (FPLC) of the active extract from a DE-52 separation was performed on a Pharmacia HR10/10 column packed with SI-17 anion-exchange resin. The column was prepared by being washed with 600 mM KH₂PO₄-KOH buffer (pH 7.45) and equilibrated in 50 mM phosphate buffer (pH 7.45). The extract was applied at 1.4 ml/min. After the protein was adsorbed, the column was washed with about 5 ml of starting buffer. Bound protein was eluted in a 300-ml gradient of 50 to 600 mM KH₂PO₄-KOH buffer (pH 7.45). Fractions were collected and assayed for PCMH activity, and protein was measured at 280 nm. Fractions containing PCMH activity were pooled and dialyzed against Tris hydrochloride buffer. The dialyzed extract was concentrated by adsorbing the protein onto a small DEAE-cellulose column (1.0 by 4.0 cm). The column was prepared by being washed with Tris hydrochloride buffer containing 400 mM KCl and equilibrated in buffer. The adsorbed protein was eluted in one step with 400 mM KCl. The eluted protein was dialyzed against Tris hydrochloride buffer and used for spectral studies.

**Isolation and characterization of the PCMH-mediated pCr metabolite pHBZ.** A reaction mixture (pH 7.6) was prepared anaerobically under \( O_2 \)-free argon gas and contained the following (per milliliter): 50 μmol of Tris hydrochloride, 4.0 μmol of PMS, 100 nmol of DCPIP, and 15 μl of DEAE-purified PCMH (16.7 μg of protein); 1.2 μmol of pCr substrate in dimethylformamide was added to start the reaction. Parallel reaction mixtures containing either no enzyme (PCMH) or no electron acceptor (PMS) served as controls to monitor any nonenzymatic oxidation of pCr. The reaction mixtures were incubated at 25°C and stopped after 30 min by being acidified to pH 2 with 0.4 N HCl. The mixtures were extracted with 2 volumes of diethyl ether, evaporated to dryness under a stream of argon gas, and suspended in 0.5 volume of high-pressure liquid chromatography (HPLC) solvent (60% methanol-40% water). The concentration of pHBZ product in the extract of the reaction mixtures was quantified by HPLC (5) with authentic external standards. The HPLC peak coeluting with the pHBZ standard was collected from the chromatographed extract, and its mass spectrum confirmed the identity and structure of the pHBZ metabolite.

In addition, the possible role of pyridine or flavin cofactors in the PCMH-mediated oxidation of pCr was investigated. Reaction mixtures contained (per milliliter) NAD, NADP, flavin mononucleotide (FMN), or flavin adenine dinucleotide (FAD) (0.4 μmol), 160 μg of protein, and 1.2 μmol of pCr substrate. Reactions with PMS but no cofactors served as positive controls; negative controls contained either no enzyme or no electron acceptor. After 20 min of incubation at 25°C, the reactions were stopped and analyzed by HPLC.

**Substrate specificity of PCMH.** A suite of pCr analogs listed in Table 1 was tested to determine the substrate range and respective affinities to the PCMH enzyme. To determine specific activity, we tested each compound \( (n \geq 2) \) at saturating concentrations (1.2 to 4.0 mM), using the colorimetric DCPIP-PMS assay for PCMH activity. Controls for background activity included reactions with either no enzyme or no substrate. Those substrates eliciting PCMH activity were also assayed at a concentration range of 0.01 to 4.0 mM to establish their relative affinities (i.e., \( K_m \)) for the enzyme.

**Oxidation of pHBZ in cell extracts.** Further enzymatic oxidation of the PCMH-mediated pCr metabolite, pHBZ, to the pOHB end product in the PC-07 pathway was measured in cell extracts. Duplicate anoxic reaction mixtures (pH 7.6) at 25°C contained (per milliliter) 40 μmol of Tris hydrochloride, 0.68 mg of protein, and 1.2 μmol of pHBZ. Addition of substrate (pHBZ) initiated the reaction. Samples were removed at timed intervals (up to 300 min), acidified with HCl to pH 2, and extracted with diethyl ether. Extracted pOHB was confirmed and quantified by HPLC with authentic standards as described before (5). Enzyme (pHBZ dehydrogenase [BZD]) activity was determined by measuring the amount of pOHB formed from pHBZ per milligram of protein. Control reactions received no enzyme.

The effect of redox was determined by assaying for BZD activity under aerobic and anaerobic conditions. The latter
were achieved chemically (3.5 mg of cysteine per ml of reaction mixture) or enzymatically (3.0 mg of glucose oxidase, 1.5 mg of catalase, and 2.0 mg of glucose (GOX) per ml of reaction mixture [9]). Although the enzymatic means (GOX) of scavenging O₂ is self-contained, controls included boiling the reaction mixtures containing GOX enzymes for 10 min before adding PC-07 cell extract to ascertain whether the GOX enzyme reductants interfered with BZD activity.

The effect of exogenous pyridine or flavin cofactors on the BZD activity of the cell extract was tested in similar anaerobic reaction mixtures which contained 0.4 μmol of one of the following electron acceptors per ml: NAD, NADP, FMN, or FAD.

**Effect of nitrate on PCMH and BZD activities in cell extracts of PC-07.** Duplicate anaerobic reaction mixtures were prepared under argon gas and contained the following (per milliliter): 50 μmol of Tris hydrochloride (pH 7.6); 0.68 mg of protein (as cell extract); 3.5 mg of cysteine; GOX O₂-scavenging system; and 1.2 μmol of substrate (pCr or pHBZ), with or without nitrate (2.0 mM). The reaction was started by substrate addition and quantitated by measuring pOHB formation. Incubation was at 25°C for 300 min. Samples were acidified and extracted with diethyl ether, and pOHB was quantified by HPLC. Nitrate reduction was determined by measuring the remaining nitrate concentration in nonextracted aqueous samples by HPLC (8).

**Enzyme and protein assays.** PCMH was assayed colorimetrically by a modified method of Hopper and Taylor (15). Reaction mixtures contained the following: PMS, 2.2 μmol; DCPIP, 100 μmol; pCr, 1.2 μmol (30 μl of a 40 mM solution in dimethylformamide); enzyme; and 50 mM Tris hydrochloride buffer (pH 7.6) to a final volume of 1.0 ml. The reaction was initiated by the addition of substrate (pCr) and could be performed under aerobic or anaerobic conditions. The rate of DCPIP reduction was monitored at 600 nm. PCMH activity is expressed in units defined as the amount of enzyme required to reduce 1.0 μmol of DCPIP per min. The molar extinction value for DCPIP at 600 nm is 21 000 (3). In cell extracts, PCMH and BZD activities were assayed by measuring product (pOHB) formation. Reaction mixtures were acidified with 0.4 N HCl, extracted with 2 volumes of diethyl ether, and analyzed by HPLC.

Protein was estimated by the method of Bradford (7), in which bovine serum albumin served as the standard.

**Analytical procedures.** Quantitation of nitrate and substrates was by reverse-phase HPLC (Beckman 334 liquid chromatograph) or UV spectrophotometry (Shimadzu UV-200 spectrophotometer) as described previously (4). Spectral studies were recorded on a Amino DW-2a spectrophotometer.

Mass spectral analysis was performed on a VG 70-250 instrument by direct insertion by ceramic probe at 300°C, using a double-focusing magnetic sector with electron ionization at 56 keV.

**RESULTS**

**Partial purification of PCMH.** The partial purification of PCMH yielded an approximate 100-fold purification after chromatographic separation by FPLC (Table 2). The apparent increase in total activity after DEAE separation may be due to a high background or to possible competing reactions in the cell extract. Figures 1a and b present the elution profile of protein and PCMH activity after DEAE-cellulose (e.g., DE-52) chromatography and FPLC, respectively. Although care was initially taken to stabilize the protein and minimize exposure of the extracts to O₂ by adding diethiothreitol (1 mM) and to protect the protein from ambient temperatures by chromatographing at 4°C, this proved to be unnecessary. PCMH and BZD in cell extracts, as well as the partially purified PCMH, are stable at room temperature and to O₂; PCMH maintains greater than 70% activity after storage at −20°C for 4 months. Further separation by S-200 molecular sieve chromatography and then electrophoresis of the native and reduced protein indicated that the protein is not homogeneous. PCMH prepared from PC-07 grown anaerobically in the presence of pCr exhibited two identical elution profiles, specific activity, and affinity (Kₘ) as the enzyme from anaerobically grown cells, thus suggesting that the PCMH is present under both conditions and is a component of both aerobic and anaerobic pathways for pCr metabolism by the PC-07 isolate.

The difference spectrum of PCMH from PC-07 was obtained by using the substrate, pCr, to reduce the partially purified PCMH which had been initially oxidized with hydrogen peroxide. The spectrum is typical of a heme-containing protein (α, β, and γ peaks at 553, 524, and 420 nm) and contains a trough at 450 nm, providing further evidence for the presence of a flavin moiety. Addition of sodium dithionite crystals did not alter the spectrum, indicating complete reduction of the chromophore by pCr.

**Substrate specificity and affinity for PCMH.** The data presented in Table 1 demonstrate a limited substrate range for the partially purified PCMH. The enzyme requires a para-substituted hydroxyl group to oxidize the alkyl substituents. From the respective specific activities obtained, it is apparent that additional ring substituents or an increase in chain length of the oxidizable alkyl group (e.g., ethyl or propyl) decreased the rate of enzyme activity. These data coincide well with earlier reports on the range of substrates metabolized by whole cells of the PC-07 isolate (I. D. Bossert and L. Y. Young, IVth International Symposium on Microbial Ecology, Ljubljana, Yugoslavia, 24 to 29 August 1986) and indicate that the reported activities are characteristic for the enzyme and not due to physical constraints such as poor solubility, partitioning in aqueous media, or transport into the cells.

The Kₘ and V₅₀ values reported in Table 1 are derived from double-reciprocal plots. The specific activity of PCMH for each substrate was tested at saturating concentrations by the PMS-DCPIP colorimetric assay. The data show that the highest affinity and rate of reaction demonstrated by the PCMH enzyme is toward pCr. The metabolite, p-hydroxybenzyl alcohol, demonstrated a slightly lower affinity and rate which may be an artifact or due to unfavorable partitioning in aqueous solutions. Results with 2,4-DMP indicated that the enzyme has a fivefold greater affinity to it than to pCr, yet 2,4-DMP yielded an approximately threefold slower rate. The rate is similar to that of 4-methylcatechol, a DMP analog which possesses a hydroxyl group in place of the methyl at the 2-position. The reason for this anomaly is
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Identification of product formed from pCr by PCMH. When pCr was oxidized by PCMH, only one major product (\(\lambda_{\text{max}}\) of 260 nm) was detected. This observation led to the tentative identification of the reaction product as pHBZ. This was further substantiated by the coelution of the reaction product with authentic pHBZ during HPLC analysis. Figure 2 shows that the fragmentation pattern of the metabolite formed from pCr by PCMH is in close agreement with that of authentic pHBZ, with corresponding major ions at 122, 93, 65, and 39 m/e.

Previous studies with whole cells have shown that PC-07 utilizes an anaerobic oxidative pathway for the metabolism of pCr to pOHB. p-Hydroxybenzyl alcohol and pHBZ have been shown to be intermediates in this pathway. The results obtained with PCMH, including the substrate range of the enzyme (Table 1), demonstrate that pHBZ is the product of the PCMH-mediated oxidation of pCr. PCMH therefore appears to be a multifunctional enzyme which catalyzes two successive oxidation steps in the PC-07 pathway.

Enzyme activity and role of nitrate in cell extracts. The oxidation of either pCr or pHBZ to pOHB by the PC-07 pathway was used to assess the respective PCMH and BZD enzyme activities in cell extracts. The latter enzyme activity completes the PC-07 pathway by mediating the oxidation of the pHBZ intermediate to the pOHB end product. The data presented in Table 3 show that both enzymes were active in anoxic cell-free reaction mixtures. pCr oxidation to pOHB (which requires the activity of both PCMH and BZD enzymes) occurred only when nitrate was available to serve as the electron acceptor. HPLC analysis of the reactions demonstrated loss of nitrate (0.71 mM for pCr; 0.83 mM for...

FIG. 1. Protein purification of PCMH from PC-07. (A) Elution profile from DEAE-52 anion-exchange chromatography of cell extract. (B) FPLC anion-exchange chromatography of pooled DEAE eluate exhibiting PCMH activity. A detailed protocol is presented in the text.

Unclear. Note that pHBZ is not a substrate for PCMH. This product of the PCMH reaction is oxidized by BZD, the activity of which was confirmed here in cell extracts.

Enzyme activity and role of nitrate in cell extracts. The oxidation of either pCr or pHBZ to pOHB by the PC-07 pathway was used to assess the respective PCMH and BZD enzyme activities in cell extracts. The latter enzyme activity completes the PC-07 pathway by mediating the oxidation of the pHBZ intermediate to the pOHB end product. The data presented in Table 3 show that both enzymes were active in anoxic cell-free reaction mixtures. pCr oxidation to pOHB (which requires the activity of both PCMH and BZD enzymes) occurred only when nitrate was available to serve as the electron acceptor. HPLC analysis of the reactions demonstrated loss of nitrate (0.71 mM for pCr; 0.83 mM for...
pHBZ). The amount of product formed (6.5 μM pOHB) from pCr was approximately threefold less than that formed from pHBZ, which is the substrate for BZD (21.0 μM pOHb formed). The observed differences in the amount of product (pOHB) formed by either substrate under identical reaction conditions suggest that the initial oxidation of pCr to pHBZ (mediated by PCMH) is a rate-limiting step in the pathway. Furthermore, the addition of NAD, NADP, FAD, or FMN cofactor did not stimulate PCMH activity. This is demonstrated by the results from a separate experiment with the partially purified PCMH enzyme. Here, pCr was oxidized to pHBZ (0.12 mM) only when PMS, an artificial electron acceptor, was added to the reaction; addition of flavin or pyridine cofactors elicited no activity. The results provide good evidence that an endogenous protein other than flavin or pyridine cofactors serves as a vehicle for the electron flow from reduced PCMH to nitrate reductase.

The data presented in Table 3 further show that oxidation of pHBZ to pOHB (mediated by BZD) occurred in the unamended cell extract but was stimulated twofold by the addition of nitrate or fourfold by NAD. NADP, FAD, or FMN did not enhance enzyme activity (data not shown). The low levels of pOHb formed in the unamended extracts were likely due to endogenous electron acceptors (e.g., NAD and nitrate reductase) still active in the extract. An increase in pOHB formation upon nitrate addition demonstrated a dependence of the reaction on nitrate, which has also been shown with whole cells (data not shown). The data in Table 3 further show that when nitrate and NAD were added together with pHBZ, BZD activity was significantly greater (105.5 μM pOHB formed) than in the reaction mixtures amended with either NO3 (21 μM pOHB) or NAD (44.5 μM pOHb) alone. This apparent synergistic effect may be explained by NAD-NADH recycling via nitrate reduction in the system.

BZD activity of cell extracts in reaction mixtures amended with either mild chemical (i.e., cysteine) or enzymatic (i.e., GOX) reductants or both demonstrated a positive correlation to reduction of the solution. The redox potential of the respective reaction mixtures (as measured by reduction of resazurin or methylene blue indicator dyes) was lowest when cysteine plus GOX served as reductants. Greatest activity occurred in the most reduced reaction mixture (30.6 μM pOHB formed), whereas the unreduced reaction mixtures (Eh = 0 mV) or those containing either cysteine or cysteine plus denatured GOX (−50 mV < Eh < 0 mV) similarly exhibited lower levels of BZD activity (7.8, 6.0, and 6.0 μM pOHB, respectively). The enhanced effect of a reducing environment underscores the O2 independence of the reaction which can be mediated by a dehydrogenase.

### DISCUSSION

Previous studies in our laboratory on the anaerobic degradation of pCr by a denitrifying bacterial coculture (4) focused on the pathway for pCr oxidation coupled to nitrate reduction, as demonstrated by one strain (PC-07) of the coculture. The studies reported here demonstrated the pCr oxidizing activity in cell extracts of the PC-07 isolate and the partial purification and characterization of a PCMH enzyme which initiates metabolic attack on pCr by anaerobically
oxidizing the substrate to pHBZ, an intermediate in the PC-07 pathway. Further oxidation of pHBZ to pOHB, the end product in the pathway, appears to be mediated by an NAD-dependent dehydrogenase (BZD), whose activity is reported here in cell extracts.

As with whole cells (5), the PCMH and BZD activities in anoxic cell extracts of PC-07 were independent of molecular O₂. Incorporation of oxygen into the pCr molecule during PCMH-mediated oxidation of the methyl group most likely occurs via water addition, as in an analogous mechanism reported by Hopper (13) for the aerobic, methylhydroxylase-mediated metabolism of pCr by Pseudomonas putida. He demonstrated ¹⁸O incorporation from ¹⁸O-labeled water (13); however, molecular O₂ in these studies was the natural external electron acceptor in the pathway. For the PC-07 isolate, nitrate can serve as the external electron acceptor and is reduced to N₂ during substrate oxidation (4).

p-HBZ, the pCr oxidation product of PCMH, is also an intermediate in the overall PC-07 pathway initially reported for whole cells (5); further oxidation of pHBZ to pOHB has now been confirmed in anaerobic cell extracts. The reaction requires no molecular O₂. In fact, activity increased by lowering the redox potential (~50 mV) of the reaction mixture. Nitrate is the terminal electron acceptor and was reduced during substrate oxidation in the cell extracts. However, the amount of nitrate reduced versus the amount of substrate oxidized was considerably higher than the expected stoichiometric proportions, as has been demonstrated with whole cells (5; unpublished data). This may be due to endogenous activity in the cell extracts.

BZD activity appears to be NAD dependent. This is in contrast to results reported by Keat and Hopper (16) for aerobic Pseudomonas strains which oxidize pHBZ to pOHB via an NAPD-dependent dehydrogenase. The PC-07 BZD enzyme exhibited no increase in activity in the presence of NAPD, thus suggesting that different dehydrogenase enzymes and perhaps different metabolic modes function in the respective aerobic and anaerobic isolates.

An increase in pOHB formation in cell extracts was observed with pHBZ as the substrate compared with that with pCr as the substrate. This corroborates previous studies with whole cells and underscores that the PCMH reaction appears to be the rate-limiting step in the anaerobic oxidation of pCr to pOHB.

The partially purified PC-07 PCMH appears to be a flavocytochrome. Although this is preliminary until spectra of a purified protein are obtained, the evidence gathered thus far shows that the PCMH possesses an oxidizable hemochromagen characteristic of cytochrome c enzymes. Its difference spectrum, reduced by its substrate pCr, indicates a flavin component. The PC-07 PCMH is a multifunctional enzyme which mediates two successive oxidations and exhibits a narrow substrate specificity. It requires para hydroxyl substitution for oxidation of the aromatic alky group. Of those substrates tested, pCr elicits the greatest specific activity to PCMH, suggesting that pCr, a naturally occurring cresol (6, 11, 18) is the natural substrate for the enzyme.

Results indicated that the PC-07 PCMH is not dependent on pyridine or flavin cofactors and therefore requires another, as yet unidentified, electron carrier protein. A likely candidate is azurin, a small copper-containing protein (1) determined to be the natural electron acceptor in aerobic Pseudomonas strains displaying PCMH activity (10, 14). Preliminary results with this purified azurin (courtesy of D. Hopper) indicate that the protein from aerobic strains is not the natural electron acceptor for the PC-07 PCMH. However, further studies are under way to determine whether a similar but species-specific azurin is functional in the PC-07 denitrifying strain.

The anaerobic oxidation of pCr by PC-07 is intriguingly similar to that already extensively studied and characterized in aerobic Pseudomonas isolates (15). Initial studies with whole cells of the denitrifying isolate PC-07 established the role and pathway of an oxidative strategy under anaerobic conditions in which nitrate is the terminal electron acceptor (5). The apparent similarities are not superficial; both aerobic and anaerobic systems demonstrate parallel nonoxygenase-mediated oxidative mechanisms catalyzed by comparable multifunctional PCMH enzymes. Subsequent reports suggest the presence of similar strategies in bacteria from other anaerobic environments. Bak and Widdel (2) have recently described a new isolate which degrades both pCr and pOHB in pure culture under sulfate-reducing conditions. The ability to utilize both these compounds suggests that pCr is oxidized to pOHB (as in the PC-07 pathway) before ring fission. Further evidence for pCr oxidation to pOHB by sediment enrichments under sulfate-reducing conditions was recently demonstrated by Smolenski and Sulfita (17) with detection of the respective hydroxybenzyl alcohol and hydroxybenzaldehyde intermediates in the pathway. In addition, Gricic-Galic (12) has suggested that a similar metabolic mode may operate in a toluene-degrading methanogenic consortium.

From the available evidence, it appears then that the anaerobic oxidation of pCr is not limited to any oxidation state. Rather, this strategy which is mediated by PCMH enzymes may have been conserved during evolution and remains a viable mechanism for substrate oxidation under a broad range of redox environments.

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LITERATURE CITED

2-aminobenzoate (anthranilic acid) by denitrifying bacteria.


