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ARTICLE *in* PROTEIN EXPRESSION AND PURIFICATION · FEBRUARY 2008

Impact Factor: 1.7 · DOI: 10.1016/j.pep.2007.09.007 · Source: PubMed

Published in final edited form as:
Protein Expr Purif. 2008 January ; 57(1): 9–16. doi:10.1016/j.pep.2007.09.007.

Soluble Expression and Purification of the Oxidoreductase Component of Toluene 4-Monooxygenase

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Abstract

Toluene 4-monooxygenase (T4MO) is a member of the bacterial multicomponent monooxygenases, an enzyme family that utilizes a soluble diiron hydroxylase to oxidize a variety of hydrocarbons as the initial step in their metabolism. The hydroxylases obtain reducing equivalents from NAD(P)H via an electron transfer chain that is initiated by an oxidoreductase containing an N-terminal ferredoxin domain and C-terminal flavin- and NAD-binding domains. T4moF, the NADH oxidoreductase of T4MO, was expressed as a soluble protein in *Escherichia coli* BL21(DE3) from the pUC-derived expression vector pRS205. This vector contains a lac promoter instead of a T7 promoter. A three step purification from the soluble cell lysate yielded ~1 mg of T4moF per gram of wet cell paste with greater than 90% purity. The purified protein contained 1 mol of FAD and 2 mol of Fe per mol of T4moF; quantitiative EPR spectroscopy showed ~1 mol of the $S = ½$ signal from the reduced [2Fe-2S] cluster per mol of T4moF. Steady state kinetic analysis of *p*-cresol formation activity treating T4moF as the variable substrate while all other proteins and substrates were held constant gave apparent K_M- and apparent k_{cat}-values of 0.15 μM and 3.0 s^{-1}, respectively. This expression system and purification allows for the recovery of the soluble oxidoreductase in yields that facilitate further biochemical and structural characterizations.

Keywords

bacterial multicomponent monooxygenase; diiron enzyme; oxidoreductase; iron-sulfur; flavoprotein

Introduction

Toluene 4-monooxygenase (T4MO) from *Pseudomonas mendocina* KR1 is a multicomponent hydroxylase that catalyzes the O_2 and NADH dependent hydroxylation of toluene to form p-cresol with high regiospecificity [1,2]. The T4MO complex is encoded by a 4.7 kb gene cluster, *tmo*A-F [3]. The six genes in this cluster encode a diiron hydroxylase (T4moH), a cofactor-less effector protein (T4moD), a soluble Rieske type ferredoxin (T4moC), and an NADH oxidoreductase (T4moF) [1,3].

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Toluene 4-monooxygenase is a member of the bacterial multicomponent monooxygenase (BMM) family. The BMMs utilize a soluble terminal hydroxylase to oxidize a variety of hydrocarbons as the initial step in their metabolism [4]. The BMM family has been divided into four groups: soluble methane monooxygenases (MMO), alkene monooxygenases, phenol hydroxylases, and alkene/aromatic hydroxylases [4]. Members of this enzyme family have either three or four protein components. All members possess a terminal hydroxylase and a cofactor-less effector protein. The three component BMMs reduce the hydroxylase via an NAD(P)H oxidoreductase containing an N-terminal chloroplast-type ferredoxin domain and C-terminal domains with flavin- and NAD-binding sites. The four component monooxygenases have an additional soluble Rieske-type ferredoxin that serves as an intermediate electron carrier between the oxidoreductase and the diiron hydroxylase. T4MO is a member of the of four component alkene/aromatic hydroxylase group of BMMs [3].

The hydroxylase (T4moH), effector protein (T4moD) and Rieske-type ferredoxin (T4moC) of the T4MO system have been purified and extensively characterized [2,5-13]. In contrast, study of T4moF has been limited by difficulties in obtaining soluble expression, with subsequent limits on the ability to obtain purified protein. Here, we report a protocol for the soluble expression and purification of T4moF. Biochemical and biophysical characterizations of the purified protein showed the stoichiometric presence of FAD and a [2Fe-2S] cluster. Furthermore, the apparent k_{cat} and apparent K_M for T4moF with the reconstituted T4MO complex were 3.0 s^{-1} and 0.15 μM, respectively, for the oxidation of toluene to p-cresol. This work provides a basis for a more detailed examination of the interactions of T4moF with other proteins of the T4MO complex.

Materials and methods

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Expression vectors

Fig. 1 shows the arrangement of promoter and genes present in the expression vectors described in this study. The vector T4moF pET3a was made by standard restriction and ligation cloning after PCR amplification of the *tmo*F gene to include 5′ NdeI and 3′ HindIII restriction sites. The T4moF pET15b vector was made in a similar manner by incorporation of 5′ NcoI and 3′ BamHI restriction sites. Vector pT4moABEF was made from pRS184f [7] by digestion with KpnI, which removed 531 bp spanning between the *tmoc* and *tmod* genes, and re-ligation to generate a non-functional *tmoc-tmod* fusion.

Protein expression

Vector pT4moABEF was transformed by heat shock into *E. coli* BL21(DE3) (Novagen, Madison, WI). Transformed cells were allowed to incubate overnight at 37 °C on Luria-Bertani agar plates containing 200 μg/mL of ampicillin. A starting inoculum for the fermenter was prepared by placing a single colony into 2 mL of Luria Bertani medium containing 200 μg/mL of ampicillin. The 2 mL culture was incubated at 37 °C with shaking at 250 rpm until it reached an OD_{600} between 0.5 and 1 (this typically occurred after ~8 h). Next, 1 L of Luria Bertani medium was inoculated with 500 μL of the 2 mL culture and incubated overnight (~12 to 14 h) at 25 °C with shaking at 225 rpm. When the 1 L culture had reached an OD_{600} between 0.5 and 1, the 1 L culture was added to 9 L of sterilized Luria Bertani medium in a Bioflo 3000 fermenter (New Brunswick Scientific, New Brunswick, NJ) at 37 °C with agitation at 250 rpm. No ampicillin was added to the culture. The dissolved O_2 content was allowed to decrease to 20% of air saturation, and after that the agitation was allowed to increase to maintain the dissolved O_2 content at 20% of air saturation. At an OD_{600} ~3, the temperature was decreased

to 25 °C and 10 mL of 1 M IPTG (Fisher Scientific, Pittsburgh, PA), 20 g of Casamino acids (Fisher Scientific), and 10 mL of a 10 mM riboflavin solution were added. After induction of protein expression, the culture growth was allowed to continue for 4 h and the OD_{600} reached ~5. The cells were recovered by centrifugation at 4500 × *g* for 25 min at 4 °C in a JS-4.2 rotor and an Avanti™ J-HC centrifuge (Beckman Coulter, Fullerton, CA). This expression protocol generally yielded 5 to 6 g of wet cell paste per liter of culture medium. The cell paste was kept at -80 °C until needed.

Protein purification

The cell paste (50 g) was thawed and re-suspended in 100 mL of buffer A [25 mM MOPS, pH 7.5, containing 80 mM NaCl and 2% (w/v) glycerol] and was sonicated on ice at maximum intensity with a Model 550 sonicator (Fisher Scientific) with a program of 10 s on, 30 s rest to give a total sonication time of 4 min. The temperature of the lysate was maintained below 10 °C. The sonicated cell suspension was centrifuged at 48000 × *g* and at 4 °C in a JA-30.50 rotor and Avanti J30-I centrifuge (Beckman Coulter) for 45 min. The supernatant was then diluted two-fold with buffer A and applied to a 45 mm × 250 mm DEAE Sepharose column (GE Healthcare, Piscataway NJ) at 40 cm/hr. The column was washed with 1 column volume of buffer A and T4moF was then eluted in a 1200 mL linear gradient of 80 to 350 mM NaCl in buffer A at 40 cm/hr. The fractions containing a brownish orange color, indicative of the flavin and iron sulfur cluster of T4moF, eluted at ~200 mM NaCl. These fractions were assayed for activity using the T4MO-catalyzed oxidation of nitrobenzene to p-nitrophenol (described below). Pooled fractions were also assayed for the conversion of toluene to p-cresol by gas chromatography [7].

The pooled fractions were concentrated using a Centricon YM-10 concentrator (Millipore, Billerica, MA) to ~4 mL and loaded onto a Superdex S-75 size exclusion column (GE Healthcare) equilibrated in buffer B [25 mM MOPS, pH 7.5, containing 100 mM NaCl and 2% (w/v) glycerol] at 20 cm/min. The colored fractions were assayed and pooled as described above.

The fractions pooled from the S-75 chromatography were diluted 2-fold with buffer C (same composition as buffer A, but with pH 7.0) and applied to a 16 mm × 10 mm DEAE Sepharose (GE Healthcare) at 150 cm/h. A linear gradient of 80 to 300 mM NaCl in Buffer C was used to elute T4moF at 40 cm/h, which again eluted at ~200 mM NaCl. The colored fractions were assayed and pooled as described above.

Activity assays

The electron transfer activity of T4moF was determined by reconstitution of the hydroxylation activity of the T4MO complex according to established protocols [7]. Individual fractions from the chromatography steps were assayed monitoring the conversion of nitrobenzene to p-nitrophenol. Briefly, 10 μL aliquots of the T4moF fractions were added to 250 μL reaction mixtures consisting of 50 mM phosphate buffer, pH 7.5, with 8 μM T4moC, 8 μM T4moD, 4 μM T4moH, 3 mM NADH and a saturating level of nitrobenzene (apparent K_M ~ 10-30 μM, [2]). The presence of active T4moF was indicated by an increase in absorbance at 410 nm corresponding to the production of p-nitrophenol as detected on a Spectra Max 384 (Molecular Devices, Sunnyvale, CA) plate reader.

The pooled fractions were further analyzed for the conversion of toluene to p-cresol using a variation of the previously described method [7]. First, multiple assays were performed with serial dilution of the T4moF fractions in order to assure that the rate of observed product formation was linearly dependent on the amount of T4moF added (addition of excess T4moF would cause an underestimation of the specific activity). The toluene oxidation reactions were

performed in a Teflon-sealed reaction vial, initiated by addition of the T4moF aliquot, and were incubated in a reciprocating water bath at 26 °C. Endpoint samples were taken after 2 min and quenched in an equal volume of 2 M HCl saturated with NaCl. The quenched samples were extracted with chloroform containing 50 μM methylbenzyl alcohol as an internal standard, and p-cresol was quantified by established gas chromatography methods [7].

Purified T4moF was assayed for 2,6-dichloroindophenol reduction as described previously [14]. Briefly, a small amount of T4moF (100 nM) was added to a reaction with 50 μM of the redox dye and 200 μM of NADH in reaction buffer. The reactions were continued for 30 s at 15 °C. Reactions without enzyme were performed to correct for background reduction.

Flavin determination and quantification

The flavin was released by heating a sample of purified T4moF (5 nmol) at 95 °C for 5 min. The denatured protein was pelleted by centrifugation in a microcentrifuge and the supernatant was further clarified using a YM-10 spin column (Millipore, Bellerica, MA). HPLC analysis was performed using a Shimadzu SCL-10A HPLC (Shimadzu, Columbia, MD) and a 100 mm × 4.6 mm C-18 column (ACE, Aberdeen, Scotland). The flavin was isolated by isocratic chromatography in a 30:70 mixture of methanol:water containing 5 mM ammonium acetate, pH 6.0, at a rate of 0.5 mL/min [15]. In this separation method, FAD, FMN, and riboflavin had retention times of 3.6, 6.0, and 10.2 min, respectively, and the flavin obtained from the protein sample had a retention time of 3.6 min. The amount of FAD obtained from T4moF was quantified by comparison of the sample peak intensity to a standard curve prepared from authentic FAD.

Mass spectral analysis

An in-gel tryptic digest was performed by the Mass Spectrometry facility of the University of Wisconsin Biotechnology Center. Mass spectrometry was performed on a 4800 MALDI TOF/TOF instrument (Applied Biosystems, Foster City, CA).

Electrophoretic analysis

Conventional denaturing polyacrylamide gel electrophoresis was performed as previously described [16]. Capillary electrophoresis was performed using a Caliper LC90 LabChip (Caliper Life Sciences, Hopkinton, MA). Samples were prepared by combining 7 μL of denaturing solution with 2 μL of protein sample. The sample was heated at 100 °C for 5 min, 35 μL of water was added to the sample, and then the sample was loaded onto the protein chip. Sample sizes were compared to a molecular weight ladder ranging from 9.4 to 119.4 kD.

Iron quantification

The total iron content of T4moF was measured by the tripyridyl-S-triazine assay [17]. The protein sample (750 μL of 30 μM T4moF) was added to 375 μL of 2 M HCl and 375 μL 20% (w/v) trichloroacetic acid in a 1.7 mL microcentrifuge tube. The solution was vortexed and then centrifuged to remove precipitated protein. The supernatant (750 μL) was added to 300 μL of 1 mM tripyridyl-S-triazine containing 25% (w/v) ammonium acetate and 2.5% (w/v) hydroxylamine hydrochloride. The color was allowed to develop for 5 min before measuring the absorbance at 596 nm. The absorbance of the sample was compared to a standard curve prepared from ferrous ammonium sulfate in order to determine iron concentration.

EPR spectroscopy

X-band EPR spectra were recorded on a Bruker ESP 300E spectrometer (Bruker, Billerica, MA) equipped with an Oxford ITC503 cryostat for low-temperature measurements. The microwave frequency was calibrated by using a frequency counter (Hewlett Packard Model

5340) and the magnetic field was calibrated with a Varian NMR gaussmeter model number 929801-01 (Palo Alto, CA). A modulation frequency of 100 kHz was used to collect all EPR spectra, and all spectra were collected using non-saturating microwave power. EPR spectra were simulated and quantified using Spin Count (ver.2.2.58), created by Professor M.P. Hendrich at Carnegie Mellon University. Spin Count is available at http://www.chem.cmu.edu/groups/hendrich/.

Results

Expression of T4moF

Previous efforts on the expression of T4moC, T4moD, and T4moH (TmoA, TmoB, and TmoE polypeptides) have yielded greater than 50 mg of purified, soluble protein per liter culture medium [5]. However, this level of soluble expression has not been achieved with T4moF, which hindered the purification and subsequent characterizations and functional studies. Efforts to express T4moF from T7 promoter vectors including pET3a, pET15b, and pET28 resulted high-level expression, but with essentially all of the protein converted into inclusion bodies [18].

As part of this work, a pUC18NotI derivative vector containing the genes encoding both T4moH and T4moF was found to yield soluble expression in a stirred vessel fermenter. Fig. 1 shows the arrangement of genes in this vector, pT4moABEF. The 4209 coding region is uniquely bound by EcoRI and XmaI restriction sites. The function of the tmoC and tmoD genes was eliminated by digestion with KpnI and religation. Vector pT4moABEF contains a rho-independent transcriptional terminator immediately upstream of the ribosome binding site of *tmo*F (position 3357 to 3379), which attenuated the total amount of expressed T4moF during induction [18] and yielded a soluble protein with cofactors present, obviating the need to refold and reconstitute T4moF.

Purification of T4moF

Table 1 summarizes the purification of T4moF. After preparation of the cell-free lysate, a sequence of a first ion exchange chromatography at pH 7.5, size exclusion chromatography at pH 7.5, and a second ion exchange chromatography at pH 7.0 gave the purified protein in greater than 90% purity. The purification required 3 days to complete from the preparation of the cell lysate, and yielded ~8% of the original activity detected in the lysate. The recovery of activity suggests that T4moF was expressed to ~13% of the soluble protein in the cell, which also corresponds well with the observed fold purification calculated by the increase in specific activity during the purification (Table 1).

Electrophoresis

The electropherograms (Fig. 2) demonstrate the level of purification of T4moF provided by each step. The mass of T4moF calculated by capillary electrophoresis was 33.5 kDa, which compared well with the mass of 35983.4 Da calculated for the polypeptide. An ingel tryptic digest of purified T4moF gave 47% coverage of the primary sequence and also revealed that the N-terminal methionine was still present, consistent with minimal activity of methionine aminopeptidase when phenylalanine is the penultimate position [19].

Catalytic activity

Fractions of T4moF were quickly identified by optical spectroscopy based on the conversion of nitrobenzene into the colored product p-nitrophenol [10]. Pooled fractions of the protein were also studied for stimulation of the conversion of toluene to p-cresol by the complete enzyme complex. In the reconstituted assay, T4moF had a specific activity of 35 mU/mg (Table

1), corresponding to an apparent k_{cat} of 3.0 s^{-1} for p-cresol formation that is likely limited by reaction of T4moH, not T4moF. Additionally, T4moF has an apparent K_M of 0.15 μM in the reconstituted assay. Purified T4moF alone also reacted with dichlorophenol indolephenol at a rate of 6.2 ± 0.1 μmol/min/mg at 15 °C, a value comparable to that found in the methane monooxygenase reductase [14].

Characterization of T4moF

Absorbance spectra of T4moF are shown in Fig. 3. The oxidized protein (solid line) has absorbance maxima at 470, 390, 345, and 280 nm (latter not shown). Complete reduction yielded a spectrum (dashed line) with a shoulder centered at ~ 520 nm consistent with other known NADH oxidoreductases. The purified protein had A_{280}/A_{460} of ~3.5, which is comparable to that observed in the purified oxidoreductases from toluene o-xylene monooxygenase, pthalate dioxygenase reductase and phenol hydroxylase [20,21]. The spectrum of the oxidized form of the enzyme shown in Fig. 3 was obtained by allowing the reduced enzyme to reoxidize in the presence of air. Except for dilution caused by sample handling, this spectrum was indistinguishable from the starting material.

The flavin released from heat-denatured T4moF had an HPLC retention time identical to that of authentic FAD. This identification is consistent with previous studies that showed the activity of cell lysates containing T4moF could be stimulated when FAD was added [18]. Quantification of FAD content revealed that the purified T4moF contained 0.9 ± 0.1 mol of FAD per mol of protein. TomoF and MMOR, the NADH oxidoreductases from the related diiron enzymes toluene/o-xylene monooxygenase and methane monooxygenase, respectively, also contain FAD [20,22].

Colorimetric iron quantification revealed 1.9 ± 0.1 mol of iron per mol of protein, suggesting a stoichiometric incorporation of a [2Fe-2S] cluster into the ferredoxin domain. The EPR spectrum of fully reduced T4moF (Fig. 4) had *g*-values of 2.05, 1.95, and 1.90 with g_{ave} = 1.97. The observed *g*-values are diagnostic for $[2Fe\text{-}2S]^{1+}$ clusters [23]. The rhombic EPR spectra of reduced T4moF exhibited inhomogeneous saturation with a power at half-saturation ($P^{1/2}$) of 0.2 mW. Spin quantification of this EPR signal under non-saturating conditions indicated 0.95 spins per mol of protein, also indicating stoichiometric incorporation of [2Fe-2S] clusters within T4moF. The as-isolated protein had no EPR spectrum prior to reduction, including the *g* = 4.3 signal that is diagnostic for adventitiously bound Fe^{3+}.

Discussion

Related oxidoreductases

Fig. 5 shows a phylogenetic tree for several oxidoreductase genes encoding proteins related in structure and/or function to T4moF. One clade of these genes, encoding T4moF, TomoF, and others has a domain arrangement consisting of an N-terminal [2Fe-2S] domain and C-terminal flavin- and NAD-binding domains. The X-ray structure of BZD is the representative structure for this clade and domain arrangement (PDB ID 1KRH,[24]). The other clade, with representatives phthalate dioxygenase reductase [25] and Rv3230c [26], has N-terminal flavin- and NAD-binding domains and a C-terminal [2Fe-2S] domain. The X-ray structure of phthalate dioxygenase reductase is the representative structure for this clade and domain arrangement (PDB ID 2PIA, [25]). In one example reported so far, catalytic cross-reactivity was not observed across the clades, i.e., T4moF did not substitute for Rv3230c [26]. The cross-reactivity within the clades has not been explicitly tested.

Expression context

The soluble expression of T4moF was apparently promoted in several ways. In early tests with several different pET vectors [18], expression temperatures, and induction methods, T4moF could be expressed at levels as high as 50% of the total cellular protein, but all was insoluble. In contrast, the use of pT4moABEF gave reproducible soluble expression, albeit at a lower level. This is a pUC18-derived expression vector that has a high copy number [5], which leads to significant basal expression from the *lac* promoter [5]. Thus addition of IPTG to cultures containing pT4moABEF gave only ~2-fold in the expression level, with much of the expression arising during the 24 h period of culture scale-up; this can be contrasted to the 20- to 100-fold increase observed with the pET vectors in an ~4 h period.

Previous studies on expression from pT4moABEF demonstrated that it is not a suitable vector for overexpression of the T4moH component [5]. Thus the yield of purified T4moH from pT4moABEF (1.9 $mg \cdot g^{-1}$ of cell paste) was typically only 30% of that from pKM11 (6.2 $mg \cdot g^{-1}$ of cell paste), another pUC18-based vector. More recently, pVP58KABE, a pQE-80 derivative with a Flexi Vector cloning cassette [27,28], has been investigated for the inducible expression of T4moH. This tightly controlled vector gave a yield of purified T4moH greater than 12 mg·g of cell paste [N. Elsen and B. G. Fox, unpublished results].

The construction of pT4moABEF placed the *tmo*F gene distant from promoter (~4 kb in the 3′ direction). Moreover, a natural rho-independent terminator immediately before the start codon of the *tmoF* gene attenuated the expression [3]. The expression medium was supplemented with Casamino acids and riboflavin; other experiments have shown this medium has sufficient iron to support cofactor incorporation at this cell density [5]. The expression was carried out at 25 °C, and the expression was terminated at low cell density. Individual cases where each of these changes helped to promote soluble expression have been reported previously [29,30].

It has been recognized that co-expression of interacting proteins can give rise to enhanced solubility and folding [10], and it is possible that weak interactions between T4moF and T4moH may have contributed to the enhanced solubility observed from pT4moABEF. However, kinetic characterizations indicate that T4moF has its primary interaction with T4moC [31], not with T4moH, and T4moC is not expressed from pT4moABEF in a functional form. Thus expression below an intrinsic solubility limit, altered folding progress, or cofactor incorporation appears to be the most likely factors that have been favorably altered in order to obtain T4moF.

Use of LabChip for electrophoresis analysis

Denaturing polyacrylamide gel electrophoresis and denaturing capillary electrophoresis were used to evaluate protein samples from each step of the purification for purity. Fig. 2 shows the electropherograms obtained from each gel sample and a gel image constructed from the electropherograms by software. Since the background threshold on the reconstructed gel image is an easily modified, user-defined parameter, we suggest that the electropherograms, which are a direct *xy* plot of measured fluorescence intensity and time, are inherently less ambiguous representations of the purification data and should be used in preference to the reconstructed gel images whenever possible.

The electropherograms also offers significant advantages for quantification of individual protein bands and for data storage relative to slab gels. For T4moF, the correlation between the amount of protein added to the capillary electrophoresis samples as determined by standard dye binding assay and the amount estimated by the capillary electrophoresis was within 10%. Moreover, the molecular mass calculated for T4moF of 33.5 kDa was in good agreement with

the 35983.4 Da calculated from the gene sequence including the N-terminal Met residue. As a caveat, quantification efforts with some other proteins (~10%) in a structural genomics context have shown that the prediction of protein amount and mass are dependent on the protein being studied [Frederick, Blommel, and Fox, unpublished results]. However, this variability has also been observed in denaturing polyacrylamide gel electrophoresis.

Purification

In combination, the characterization and catalytic assay results indicate that T4moF obtained the required FAD and [2Fe-2S] cofactors during expression, and that these two were effectively stabilized and retained during the described purification protocol.

Recently, a purification of TomoF, the oxidoreductase of the closely related toluene/o-xylene hydroxylase was reported [20]. This purification included solubilization of the protein from inclusion bodies, refolding, and then reconstitution of the iron sulfur cluster and flavin cofactors. This successful, but labor-intensive effort, gave ~3-4 mg of protein per 10 mg of inclusion bodies prepared [20].

Purification of T4moF directly from the bacterial cell paste required 3 days to complete, and yielded ~1 mg of pure protein per gram of cell paste. Purified T4moF contained stoichiometric levels of FAD and [2Fe-2S], indicating both cofactors were incorporated during expression, and effectively stabilized and retained during the purification. Moreover, purified T4moF was capable of supplying electrons for the T4MO reaction at the maximal rate observed for steady-state turnover of the complete complex, indicating this is not the rate-limiting step during steady-state catalysis. The presented purification allows for a more detailed study of the steps preceding the rate-determining event.

Acknowledgements

The NSF Grant MCB-0316232 to B.G.F. supported this work. The Peterson Fellowship from the Department of Biochemistry, University of Wisconsin supported L.J.B. The NIH NRSA Fellowship 1 F32 GM074385 supported B.S.P. The authors thank Robert J. Steffan and Kevin McClay (Shaw Environmental, Inc.) for providing the plasmids including T4moF used to initiate this work.

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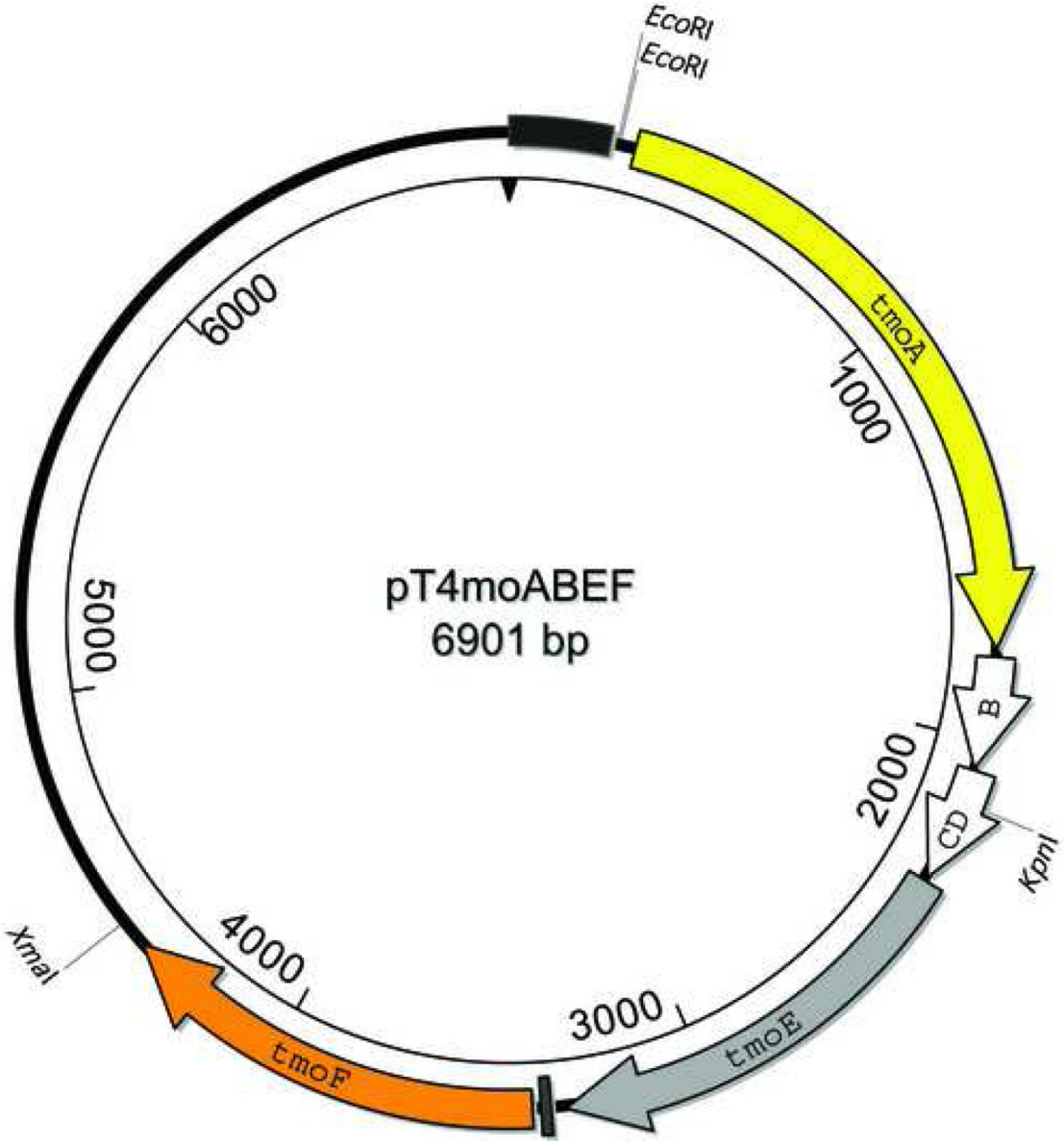

Fig. 1. Plasmid

Vector pT4moABEF used to express T4moF. Lac operon coding region from position 1 to 229 (black box), tmoA gene from 288 to 1790, tmoB from 1809 to 2063, KpnI-derived fusion of tmoC and tmoD from 2069 to 2329, tmoE from 2340 to 3323, rho-dependent terminator from 3357 to 3379 (black bar), and tmoF gene from 3399 to 4379.

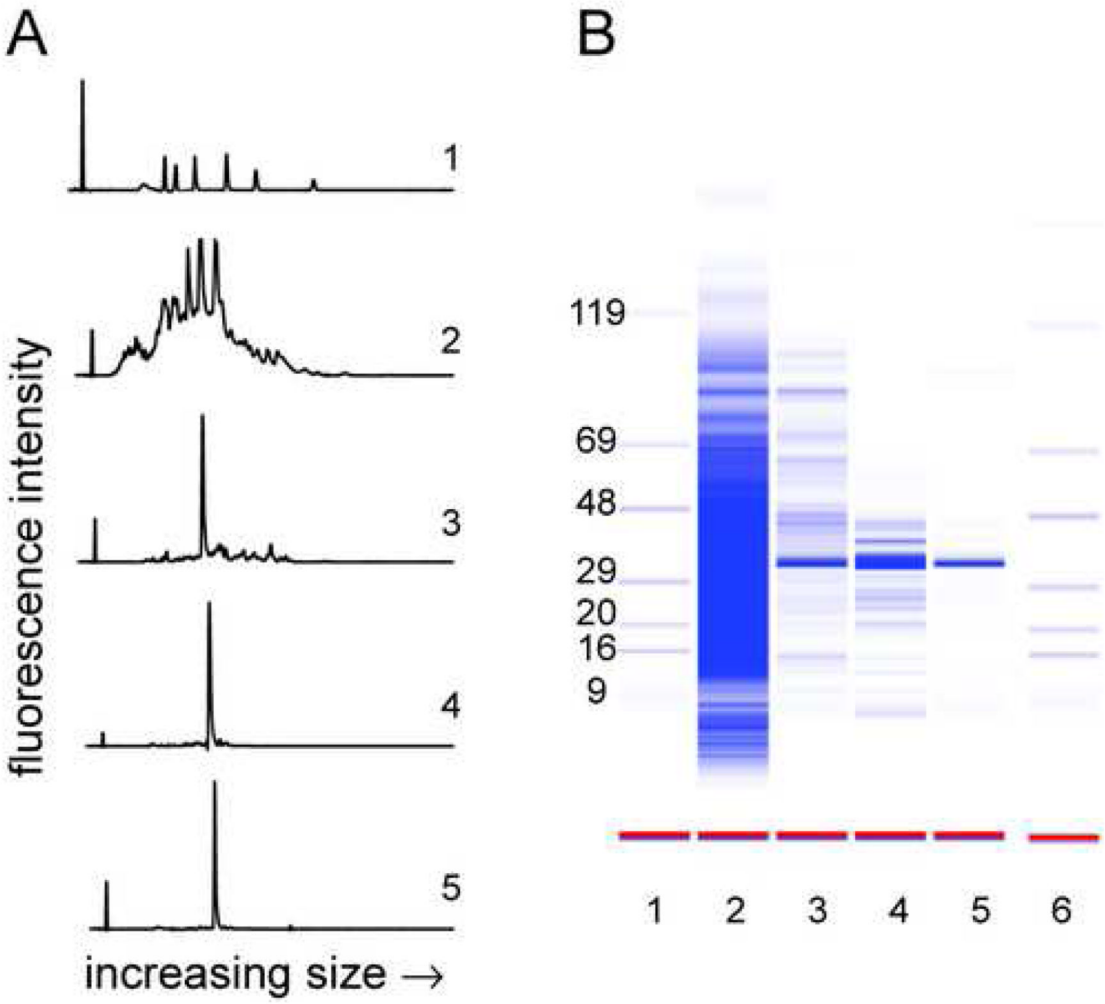

Fig. 2. Electrophoresis

An analysis of the T4moF purification using capillary electrophoresis. A, electropherograms obtained from Caliper LC90 analysis of the pooled fractions from each step of the T4moF purification. The individual electropherograms are labeled as follows: 1, molecular weight markers; 2, cell free extract; 3, pooled sample from the first DEAE column, pH 7.5; 4, pooled sample from Sephacryl S-75 column chromatography; 5, pooled sample from the second DEAE column, pH, 7.0. Integration of the peak area of the total protein signal detected from each sample allowed calculation of the percentage corresponding to the molecular weight of T4moF. These percentages are reported in Table 1. B, software conversion of the electropherograms from A into an image similar to that obtained from conventional denaturing polyacrylamide gel electrophoresis.

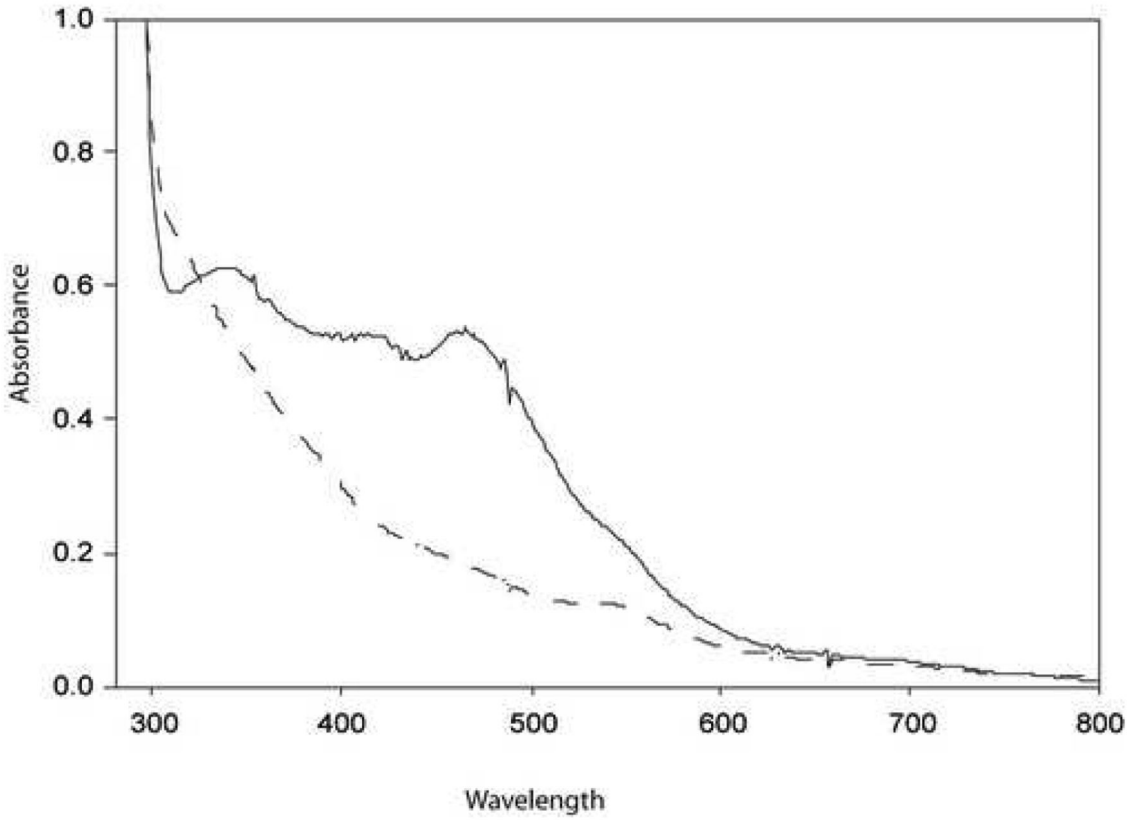

Fig. 3. Optical spectra

Optical absorption spectra of T4moF (30 μM) in 25 mM MOPS, pH 7.5, containing 80 mM NaCl and 2% (w/v) glycerol. The enzyme was reduced by the addition of an excess of buffered sodium dithionite and a catalytic amount of methyl viologen. The residual sodium dithionite and methyl viologen were then removed by gel filtration in an anaerobic chamber, and the sample was then placed into an optical cuvet and sealed under Ar. The spectrum of the reduced enzyme is shown as the dashed line. The sample was then exposed to air, and the spectrum of the oxidized enzyme, shown as the solid line, was obtained after ~10 min.

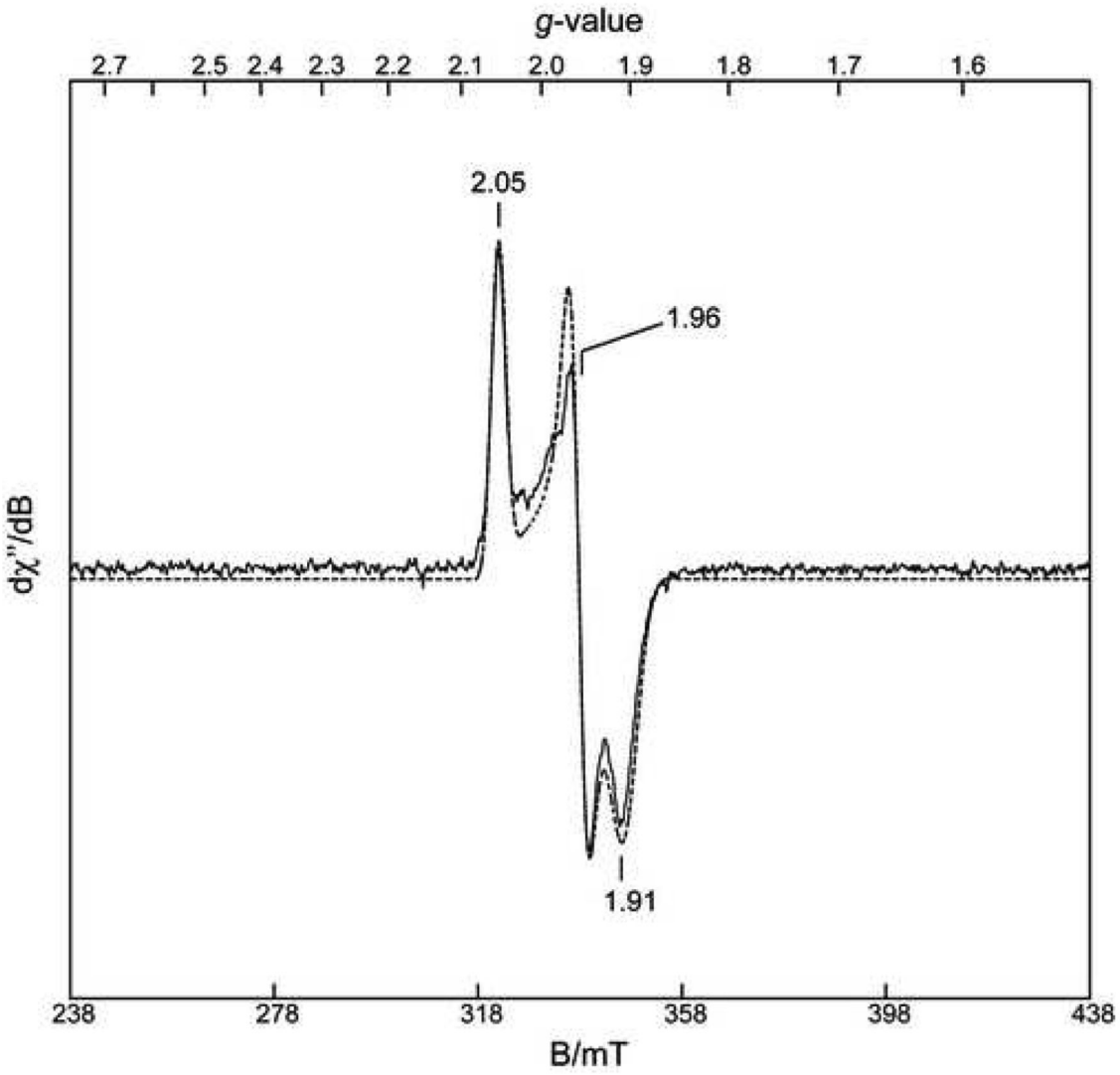

Fig. 4. EPR analysis

X-band EPR spectrum of reduced T4moF. The T4moF sample (95 μM protein) was reduced with excess sodium dithionite and methyl viologen. The sample was passed through a desalting column in a glove box to remove residual sodium dithionite and methyl viologen and placed into an EPR tube. The EPR spectrum was recorded on a Bruker ESP 300E spectrometer equipped with an Oxford ITC4 cryostat. Instrumental parameters: microwave frequency, 9.25 GHz, microwave power, 0.02 mW; modulation ampltude, 1.0 mT; temperature, 10 K, and modulation frequency, 100 kHz. A simulation (dashed line) is overlaid on the EPR spectrum of the reduced T4moF (solid line). Simulation parameters: S = 1/2, $g_{x,y,z}$ = 2.05, 1.96, and 1.90; $\sigma g_{x,y,z,}$ = 0.009,0.009, and 0.014; σ_B = 0.5 mT.

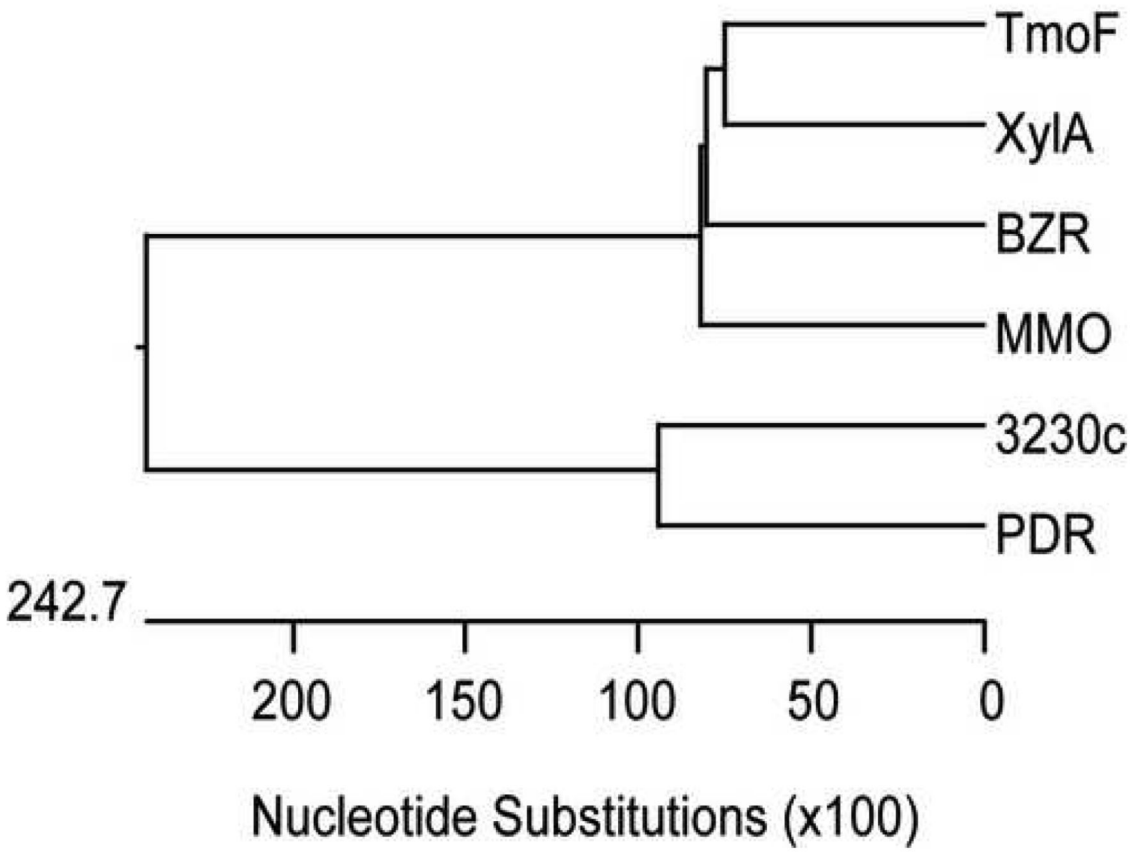

Fig. 5. Phylogenetic tree

ClustalV (PAM250) alignment of selected bacterial oxidoreductase genes. The upper clade encodes proteins with an N-terminal [2Fe-2S] domain and C-terminal flavin- and NAD-binding domains. The abbreviations are T4moF, *Pseudomonas mendocina* KR1 toluene 4-monooxygenase reductase GB: AAS66665; TomoF *Pseudomonas stutzeri* OX1 GB: AAT40436; XylA, *Pseudomonas oleovorans* xylene monooxygenase reductase GB:AAD28568; 1KRH, *Acinetobacter sp*. ADP1 benzoate dioxygenase reductase GB: AAC46438; MMO *Methylosinus trichosporium* OB3b methane monooxygenase reductase GB:CAB45257. The bottom clade encodes proteins with N-terminal flavin- and NAD-binding domains and a C-terminal [2Fe-2S] domain. The abbreviations are 3230c *Mycobacterium tuberculosis* H37Rv DesA3 reductase GB:O05875; PDR *Pseudomonas cepacia* phthalate dioxygenase reductase GB:P33164.

Table 1

Purification summary

Purification of T4moF, the NADH oxidoreductase of toluene 4-monooxygenase

Step	Total protein	Total activity	Activity recovered	Specific activity	Fold purification	Purity
	(mg)[a]	(U)[b]	%	(U/mg)		%
Cell-free lysate	8400	20400	100%	2.4	1.0	14
Anion exchange, pH 7.5	930	13600	67%	14.6	6.1	33
Size exclusion	70	1880	9%	26.8	11.2	83
Anion exchange, pH 7.0	45	1580	8%	35	14.6	92

[a] Protein concentration determined by Bradford reagent.

[b] A unit of T4moF activity is defined as the formation of 1 μmol of *p*-cresol per min in air-saturated 50 mM phosphate buffer, pH 7.5 at 298 K in the presence of optimal concentrations of the other T4MO components, toluene-saturated buffer (5.8 mM at 298 K), and NADH (0.5 mM) as determined by gas chromatography [7].

[c] The percentage contribution of the T4moF polypeptide to the total protein content of the purification samples was determined by LC90 capillary electrophoresis.