4-Toluene Sulfonate Methyl-Monooxygenase from *Comamonas testosteroni* T-2: Purification and Some Properties of the Oxygenase Component

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Comamonas testosteroni T-2 synthesizes an inducible enzyme system that oxygenates 4-toluene sulfonate (TS) to 4-sulfobenzyl alcohol when grown in TS-salts medium. We purified this TS methyl-monooxygenase system (TSMOS) and found it to consist of two components. A monomeric, iron-sulfur flavoprotein (component B), which has been shown to act as a reductase in the 4-sulfobenzoate dioxygenase system of this organism (H. H. Locher, T. Leisinger, and A. M. Cook, *Biochem. J.* 274:833-842, 1991), carried electrons from NADH to component M, an oxygenase. This oxygenase had the UV-visible spectral characteristics of an iron-sulfur protein. M_s of about 152,000 for the native oxygenase and of 43,000 under denaturing conditions indicated a homotri- or homotetrameric enzyme, whose N-terminal amino acids and amino acid composition were determined. The activity of the purified enzyme was enhanced about fivefold by the addition of Fe^{2+} . In the presence of O_2 and NADH, components B and M together catalyzed the stoichiometric transformation of TS or *p*-toluate to the corresponding alcohol. The reaction was confirmed as oxygenation of the methyl group by observation of an oxygen atom from $^{18}\text{O}_2$ in carboxybenzyl alcohol. The substrate range of TSMOS included carboxylated analogs of TS (*p*- and *m*-toluates and 4-ethylbenzoate), whereas *p*-xylene, toluene, and *p*-cresol were not substrates. TSMOS also catalyzed demethylation; 4-methoxybenzoate was transformed to 4-hydroxybenzoate and formaldehyde.

Many aromatic compounds which contain an *O*-methyl or a ring methyl group are metabolized by aerobic bacteria via the initial introduction of a hydroxy function into the methyl group (9-11, 37). Three types of enzyme catalyze these initial reactions. Firstly, flavin hydroxylases introduce hydroxyl groups from water into methyl groups *para* to hydroxyl groups (*p*-cresol methylhydroxylases [18]; see also reference 26). Secondly, monooxygenases catalyze *O* demethylation (4-methoxybenzoate *O*-demethylase [5, 27]) and, as a side reaction, monooxygenation of aromatic methyl groups (3). Thirdly, monooxygenation of aromatic methyl groups is recognized. However, very little is known about the biochemistry of this third type of enzyme (10, 14, 15), although a two-component methyl-monooxygenase system for toluene (and xylenes) has been postulated from chromatographic evidence (24) and genes for a toluene (and xylene) methyl-monooxygenase have been sequenced (32).

Comamonas testosteroni T-2 utilizes the xenobiotic compound 4-toluene sulfonate (TS) as the sole source of carbon and energy for growth (21, 33). TS is metabolized by oxidation of the methyl group to form 4-sulfobenzoate (PSB), which is desulfonated by the action of the PSB dioxygenase system (PSBDOS) (21, 22) (Fig. 1). The introduction of the hydroxyl group to form 4-sulfobenzyl alcohol in crude extracts was dependent on the presence of NADH and stoichiometric amounts of O_2 , and a monooxygenase reaction was postulated (21). We recently reported the purification and characterization of the desulfonative, two-component PSBDOS of *C. testosteroni* T-2 (22). When the reductase (component B or reductase B) of this system was

combined with a distinct fraction from an anion-exchange column, TS monooxygenase activity was obtained.

We now report the purification and some properties of the oxygenase (component M or oxygenase M) of this two-component enzyme system, which we term the TS methyl-monooxygenase system (TSMOS).

MATERIALS AND METHODS

Materials. *p*-Xylene, *p*-cresol, toluates, 4-ethylbenzoate, 4-methoxybenzoate, trifluoroacetate, and Ponceau S red were from Fluka, Buchs, Switzerland. 4-Carboxybenzyl alcohol was from Sigma Chemical Co., St. Louis, Mo. $^{18}\text{O}_2$ (97% enrichment) was obtained from Medgenix GmbH, Düsseldorf, Germany. Beef liver catalase was from Boehringer GmbH, Mannheim, Germany. The sources of other chemicals and of materials for protein purification are given elsewhere (21, 22, 33).

Analytical methods. Substrate turnover was monitored by high-pressure liquid chromatography (HPLC) on reversed-phase columns (21); UV spectra were recorded in a diode array detector. Products for analysis by mass spectrometry (MS) were pre-separated in 0.1% (vol/vol) aqueous trifluoroacetic acid containing appropriate amounts of methanol. Positive chemical ionization (PCI) mass spectra were measured in a quadrupole MS (model 4500; Finnigan, San Jose, Calif.) with a direct-exposure probe. The energy setting was 40 eV, and methane was used as the reagent gas. Electron impact (EI) mass spectra were obtained on a particle beam HPLC-MS (Hewlett-Packard, Palo Alto, Calif.) (36). Samples were separated on Nucleosil-C18 reversed-phase columns (2.1 by 100 mm) with an eluent consisting of 0.5% (vol/vol) formic acid in water and a linear gradient of

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FIG. 1. Degradative pathway of TS in *C. testosteroni* T-2.

methanol (0 to 100% over 30 min, with a flow rate of 0.2 ml/min). Formaldehyde was determined colorimetrically as described by Stucki et al. (30). Protein concentrations were measured by the method of Bradford (7) with bovine serum albumin as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) was used to monitor protein purification and to estimate molecular weights under denaturing conditions. Proteins were stained with Coomassie brilliant blue (20, 22). The N-terminal amino acid sequence was determined with protein bands blotted from SDS-PAGE gels onto polyvinylidene difluoride membranes and subjected to Edman degradation (22). Blotting was performed with a Mini Trans-Blot cell (Bio-Rad, Richmond, Calif.) at 50 V (140 to 170 mA) for 1 h at 4°C. The blotting buffer consisted of 25 mM Tris base, 190 mM glycine, 20% (vol/vol) methanol, and 0.02% (wt/vol) SDS. After being blotted, proteins were stained with 0.2% (wt/vol) Ponceau S red in 3% (vol/vol) trichloroacetic acid. The amino acid compositions of de-salted proteins were determined, and the values obtained were corrected when necessary for losses during hydrolysis (22). The M_r of the native enzyme was estimated by gel filtration through a Superose 12 column (10 by 300 mm) (Pharmacia, Uppsala, Sweden) as detailed elsewhere (22).

Growth of the organism, preparation of cell extracts, and enzyme assays. *C. testosteroni* T-2 (21, 33) was grown in TS-, PSB-, or succinate-salts medium (22). Cells for the purification of TSMOS were obtained and stored as described elsewhere (22), except that PSB was replaced by TS. Cells were disrupted in a French pressure cell, and crude extract was prepared as described elsewhere (22). This extract could be stored at -20°C for at least 4 weeks without the loss of TSMOS activity; at 4°C about 20% of the activity was lost over 24 h.

TSMOS activity was routinely assayed as oxygen uptake at 30°C in a Clarke-type oxygen electrode with a 1-ml reaction vessel (22). The optimized reaction mixture contained (in 0.5 ml) 25 μ mol of potassium phosphate buffer (pH 7.0), 200 nmol of NADH, 40 nmol of FeSO_4 , and protein (with crude extracts, 750 μ g, and with purified enzyme, 20 to 50 μ g, each of oxygenase [component M] and reductase [component B]), and the reaction was started by the addition of 500 nmol of 4-ethylbenzoate (see Results for an explanation). The stoichiometry of the purified TSMOS reaction was determined as substrate disappearance and product formation by HPLC, coupled to measurements of oxygen consumption. H_2O_2 formed by uncoupling of the electron flow with some pseudosubstrates for TSMOS was detected as the liberation of oxygen upon the addition of 6,500 U of catalase to the standard oxygen uptake assay (3). The source of the hydroxyl group in the product formed from *p*-toluate was examined in an experiment in which the enzyme assay was carried out in gas-tight 10-ml vials containing defined atmospheres of nitrogen (80% [vol/vol]) and oxygen (20% [vol/vol]) either $^{16}\text{O}_2$ or $^{18}\text{O}_2$ (21). Reaction mixtures (2 ml) contained 100 μ mol of potassium phosphate buffer (pH 7.0),

1.6 μ mol of NADH, 160 nmol of FeSO_4 , and 50 to 100 μ g each of oxygenase (component M) and reductase (component B). The reaction was started by the injection of 4 μ mol of degassed substrate (*p*-toluate). After 15 min of incubation at 30°C in a shaking water bath, the reaction was stopped by precipitation of the proteins with 60 μ l of 70% (vol/vol) perchloric acid. Proteins were removed by centrifugation, and products in the supernatant fluid were monitored and purified by HPLC. Carboxylated products were separated from the eluent by extraction (twice) with equal volumes of diethyl ether, which was removed by evaporation, and the residues were dissolved in small volumes of H_2O prior to analysis by MS.

Reductase B was identified as TSMOS activity when fractions were combined with component M, and reductase B was assayed routinely at 25°C as cytochrome *c* reduction (22).

Purification of TSMOS. The procedure for TSMOS purification is in all practical respects similar to that for PSBDOS purification (22).

(i) **Step 1.** Nucleic acids were removed by precipitation with protamine sulfate as described elsewhere (22).

(ii) **Step 2.** The anion-exchange column (16 by 100 mm; Mono Q; Pharmacia) was equilibrated (4 ml/min) with buffer A (25 mM potassium phosphate buffer [pH 7.0] containing 1 mM dithiothreitol) and loaded with the supernatant from step 1 (10 ml; 400 mg of protein). Proteins were eluted with an increasing gradient of Na_2SO_4 (see Fig. 2), and 4-ml fractions were collected. No single fraction showed TSMOS activity, but activity was obtained when red-brown fractions M (no. 24 to 28) were combined with yellow fractions B (no. 34 to 38), which were known to contain reductase B (22). Fractions which showed TSMOS activity when combined with component B were pooled, and the material (crude component M) was concentrated by membrane filtration (Centriprep; Amicon, Danvers, Mass.). Fractions containing reductase B were pooled and purified to homogeneity as reported previously (22).

(iii) **Step 3.** Hydrophobic interaction chromatography was done with a phenyl-Superose column (5 by 50 mm; Pharmacia). Crude component M from step 2 was brought to 1 M $(\text{NH}_4)_2\text{SO}_4$ by the addition of 3 M $(\text{NH}_4)_2\text{SO}_4$ and, in two separate runs, was loaded onto the column, which had been equilibrated (0.5 ml/min) with buffer A containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The column was rinsed for 10 min, and the concentration of $(\text{NH}_4)_2\text{SO}_4$ was decreased to 0 M over 50 min. Fractions of 1 ml were collected and assayed for TSMOS activity in the coupled assay. TSMOS activity eluted in a symmetrical, well-separated peak at about 250 to 300 mM $(\text{NH}_4)_2\text{SO}_4$. The active fractions from both runs were pooled and concentrated.

(iv) **Step 4.** Component M from step 3 was loaded on a gel filtration column (21.5 by 300 mm; TSK G 3000 SW; Toyo Soda, Tokyo, Japan) equilibrated (3 ml/min) with buffer A containing 150 mM Na_2SO_4 . Samples of 1.5 ml were collected and assayed for TSMOS activity in the coupled assay.

TABLE 1. Purification of component M of TSMOS^a

Step	Protein (mg)	Activity (nkat)	Sp act (mkat/kg of protein)	Recovery (%)	Purification (fold)
Crude extract	309	953	3.08	100	1
Protamine sulfate treatment	303	910	3.00	95.5	0.97
Mono Q	18.8	548	29.1	57.5	9.5
Phenyl-Superose	4.8	230	47.9	24.1	15.6
Gel filtration	2.8	190	67.8	19.9	22

^a TSMOS was assayed as 4-ethylbenzoate-dependent oxygen uptake in the presence of partially purified reductase B, such that valid specific activities were obtained, as described in Materials and Methods.

The activity eluted in a single symmetrical peak at about 25 min. The active fractions were pooled, concentrated about five times, and stored at -20°C or used immediately for characterization.

RESULTS

Enzyme assay for TSMOS and specific activities. Assays for multicomponent oxygenases in crude extracts tend to display a nonlinear response to protein concentration (e.g., 1, 34) because of a lack of reductase (2, 22). The assay for TSMOS in crude extracts, however, showed an essentially linear response to protein concentration, and the addition of crude reductase B (50 to 100 μg) caused little alteration in rate ($<10\%$) or linearity. Crude extracts from TS-grown cells are therefore essentially saturated with reductase. Nevertheless, to obtain similar conditions in all assays, we always added 50 μg of crude component B. Comparison among preparations was complicated by the fact that, although the monooxygenase itself requires only 1 mol of O_2 per mol of TS, the reaction of the crude extract with TS consumes 3 mol of O_2 per mol of TS (21). Since TSMOS is more active than PSBDOS is (21), the measured oxygen uptake rates in crude extracts are less than threefold the net reaction rate with TS as the substrate. We solved this problem by exploiting the wide substrate range of TSMOS (see below) and choosing a substrate (4-ethylbenzoate) whose transformation product was not oxygenated in crude extracts. We thus had an assay system which allowed a direct comparison of valid specific activities of TSMOS among different enzyme preparations or crude extracts.

The supernatant fluid from cell extracts from *C. testosteroni* T-2 optimally grown in TS-salts medium contained highly active TSMOS (3.1 mkat/kg of protein) (Table 1) compared with extracts from PSB- and succinate-grown cells (0.2 and 0.01 mkat/kg of protein, respectively). This result confirms initial observations (21) that TSMOS is a soluble and inducible enzyme system. This high specific activity (3.1 mkat/kg of protein with 4-ethylbenzoate) (Table 1) must represent about 3.9 mkat/kg of protein with TS as the substrate (see below) or about 85% of the TSMOS activity calculated to be present in growing cells (21). It seems, therefore, to be a reasonable assumption that we are examining proteins responsible for TSMOS activity in growing cells.

Purification of TSMOS. When proteins in crude extracts were separated by anion-exchange chromatography, no single fraction was active, but three colored bands were observed (red-brown regions A and M and yellow region B). Regions A and B contained the oxygenase (A) and the reductase (B) components of PSBDOS (22). TSMOS activity

was obtained when regions M and B were combined (Fig. 2). Purified reductase B combined with fraction M showed TSMOS activity, confirming that this reductase is active in both PSBDOS and TSMOS. Whereas reductase B represented about 1% of the soluble protein in earlier work (22), we found it to be present at 5 to 10% in cells optimally induced for TSMOS but only in small amounts ($<1\%$) in cells grown solely in PSB-salts medium.

Component M, the putative oxygenase component of TSMOS, was purified by hydrophobic interaction chromatography and gel filtration. Only one protein peak was observed in the final step (data not shown), and essentially one band was observed upon SDS-PAGE (Fig. 3), although a frequent contaminant (about 2%) of M_r 34,000 was found. A typical purification of component M is shown in Table 1. About 3 mg of pure component M was obtained from about 300 mg of soluble protein (about 8 g of wet cells). Assuming no significant losses of activity upon extraction and purification, component M of TSMOS represented about 4.5% of the total soluble protein in strain T-2, consistent with the density of the relevant band in crude extracts (Fig. 3).

Physical properties of component M of TSMOS. The M_r of native component M, as determined by gel filtration, was about 152,000. Under denaturing conditions, an M_r of 43,000 was observed (Fig. 3). We presume that the native enzyme is composed of four or three subunits of identical size.

The amino acid composition of component M of TSMOS is shown in Table 2. The N-terminal amino acid sequence was Met-Phe-Ile-Ile-Asn-Asn-(Trp)-Tyr-Val-(Ala)-Ala-(Asp)-Thr-X-Ile-Pro. The parentheses indicate a questionable identification. One unique sequence was obtained, indicating that the enzyme is composed of identical subunits.

The absorption spectrum of the purified enzyme showed maxima at 560 nm (shoulder), 462 nm, and 330 nm (Fig. 4). When the enzyme was reduced by trace amounts of dithionite or by reductase B and NADH, a loss of absorption at 560 and 462 nm was observed, and a new maximum appeared at 521 nm. These characteristics are similar to those of other oxygenase components (Table 3), which are known (1, 2) or believed (22) to contain a Rieske iron-sulfur center. We thus presume oxygenase M of TSMOS to contain an iron-sulfur center.

Catalytic properties of TSMOS. The substrate specificity of purified TSMOS was examined in the oxygen uptake assay, and transformation of substrates was determined by HPLC. At least four methyl or ethyl aromatic compounds were substrates and subject to hydroxylation (Table 4). The reaction with *p*-toluate was also examined in the presence of $^{18}\text{O}_2$, and the PCI mass spectrum obtained from the product showed a protonated molecular ion at m/z 155 $[(M + H)^+]$, two mass units higher than the corresponding ion obtained from the product formed in the presence of $^{16}\text{O}_2$ or from authentic *p*-carboxybenzyl alcohol. This result demonstrates the incorporation of one oxygen atom from $^{18}\text{O}_2$ and confirms that the reaction is monooxygenation. The EI fragmentation pattern of the putative *p*-carboxybenzyl alcohol formed from *p*-toluate in the presence of $^{16}\text{O}_2$ was identical to that of authentic material (data not shown), confirming the tentative identification. TS, *p*-toluate, and 4-ethylbenzoate were converted stoichiometrically, with the consumption of 1.0 to 1.2 mol of O_2 per mol of substrate, to 1 mol of the corresponding alcohol (when a standard was available). Under NADH-limiting conditions, a ratio of 0.9:1.0 for the consumption of O_2 and NADH was obtained. We thus presume that TSMOS catalyzes the oxygenation of 1 mol of substrate with 1 mol each of NADH and O_2 to 1 mol each of

FIG. 2. Separation of the two components of TSMOS by anion-exchange chromatography. Proteins were eluted with an Na₂SO₄ gradient (---). Component M (oxygenase) (●) was located by measuring the TSMOS activity of fractions in assay mixtures containing 50 μg of crude component B (fraction no. 37). Component B (reductase) (○) was located by measuring the TSMOS activity of fractions in assay mixtures containing 80 μg of crude component M (fraction no. 26). Protein was monitored as A₂₈₀ (—).

the alcohol, NAD⁺, and H₂O. The alcohol was not subject to further transformation by TSMOS.

4-Ethylbenzoate was oxygenated to a more polar product with a UV spectrum similar to that of 4-carboxybenzyl alcohol (Table 4). The PCI mass spectrum showed a proto-

nated molecular ion at m/z 167 [(M + H)⁺], consistent with a 4-carboxyphenyl ethanol. Whether the C-1 or the C-2 carbon of the ethyl substituent was hydroxylated [forming either 1-(4-carboxyphenyl)- or 2-(4-carboxyphenyl)-ethanol] could not be definitively determined, because no reference compound was available (Table 4). Preliminary results obtained from EI fragmentation spectra did not favor hydroxylation at the terminal methyl group, because an ion at m/z 151 [(M - CH₃)⁺] was observed.

FIG. 3. SDS-PAGE of different steps during the purification of component M of TSMOS. Proteins were detected by staining with Coomassie brilliant blue. Lanes: A, crude extract (15 μg of protein); B, crude component M after the Mono Q step (4 μg); C, component M after the phenyl-Superose step (2 μg); D, purified enzyme after gel filtration (2.5 μg); E, protein markers (approximately 0.3 μg each). In lane A, the locations of the bands corresponding to the oxygenase component of PSBDOS (a) and reductase B (b) are indicated (22).

TABLE 2. Amino acid composition of component M of TSMOS

Amino acid ^a	No. of residues/enzyme subunit ^b
Asx.....	37.0
Glx.....	36.2
Ser.....	12.2
Gly.....	26.4
His.....	10.4
Arg.....	23.9
Thr.....	18.9
Ala.....	29.1
Pro.....	24.6
Tyr.....	6.9
Val.....	14.5
Met.....	3.0
Ile.....	15.9
Leu.....	28.9
Phe.....	10.5
Lys.....	16.7

^a Trp and Cys were not determined.

^b The values were calculated on the basis of a molecular weight of 43,000. The data represent the means of two determinations.

FIG. 4. Absorption spectra of component M of TSMOS. (a) Purified component M (0.8 mg/ml) in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. (b) Same sample after reduction with sodium dithionite (1 mg/ml). The same spectrum was obtained when the enzyme was reduced with 50 μ g of purified reductase B and 0.2 mM NADH instead of dithionite. We suspect that the presence of dithionite or NADH masked the features of the spectrum at wavelengths below 400 nm.

TSMOS also oxygenated 4-methoxybenzoate, resulting in O demethylation (Table 4). In contrast, apolar methyl aromatic and aliphatic compounds (*p*-xylene, toluene, and propane) were not substrates for TSMOS. Similarly, some sulfonated or carboxylated aromatic compounds, e.g., terephthalate and PSB, were not substrates (Table 4). Several compounds caused significant, substrate-dependent oxygen uptake with TSMOS but were apparently not transformed (Table 4). This phenomenon is referred to as uncoupling of the electron flow (17) and seems to be common in two-component oxygenases (e.g., 2, 3). The oxygen consumed by this process is apparently liberated as H_2O_2 , because

TABLE 3. Data from UV-visible spectra of oxygenase components of different multicomponent oxygenases

Substrate for oxygenase	Wavelength (nm) ^a					Source or reference
	Oxidized form			Reduced form		
	Max ₁	Max ₂	Max ₃	Max ₁	Max ₂	
TS	330	462	560	ND	521	This work
4-Methoxybenzoate	325	455	570(s)	412	518	5
PSB	327	467	560	430	520	22
4-Chlorophenylacetate	325	458	564(s)	423(s)	519	23
<i>o</i> -Phthalate	328	466	560(s)	434(s)	517	2
Benzoate	323	464	560(s)	ND	518	38
Benzene	326	450	550	410(s)	524	8
Pyrazon	ND	445	545(s)	410	520	29
Naphthalene	334	462	566(s)	380	520	12

^a (s), shoulder; ND, not detected.

significant amounts of oxygen were formed when catalase was added to assay mixtures which contained uncoupling substrates (e.g., 4-chlorobenzoate), whereas no H_2O_2 was detected with TS or *p*-toluate as the substrate. 4-Aminobenzenesulfonate caused uncoupling, but some substrate disappearance was found, although no significant product was detected (Table 4). It seems that compounds with a structure similar to that of the original substrate can form an enzyme-substrate complex that catalyzes the activation of O_2 , which is not incorporated into the substrate but is liberated as hydrogen peroxide (3).

Fe^{2+} was essential for the optimal activity of TSMOS. The reaction rate of the purified enzyme was increased about fivefold by the addition of Fe^{2+} to the assay mixture, whereas the activity was only doubled when Fe^{2+} was added to crude extracts. We presume that some iron is lost from the protein during purification.

DISCUSSION

TSMOS is the first monooxygenase system to be isolated that initiates the degradation of a methyl aromatic compound at the methyl group. The system is composed of two components: a monomeric 36-kDa NADH oxidoreductase containing a flavin adenine mononucleotide (FMN) and a 2Fe-2S center (22), which transfers electrons to the oxygenase component, apparently a multimeric iron-sulfur protein consisting of 43-kDa subunits. The system has many similarities with the well-characterized 4-methoxybenzoate O-demethylase system of *Pseudomonas putida* (EC 1.14.99.15) (5) and with one class of two-component dioxygenases (e.g., EC 1.14.12.7) (2). Each has a monomeric, flavin adenine mononucleotide-containing reductase whose iron-sulfur center fulfills a ferredoxin function in transferring electrons from NADH to an oxygenase; M_s are 34,000 (2), 36,000 (22), and 42,000 (5). The oxygenase is homomultimeric, with apparently two (22), three (5, 23), or four (2) subunits, each with one presumed Rieske (2Fe-2S) center per subunit. It is uncertain whether the oxygenase component of TSMOS is composed of three or four subunits. Catalytically, these two-component oxygenases frequently display the uncoupling indicated for TSMOS in Table 4 (2, 3). There appears to be more in common among these oxygenases than the separation in the EC systematics implies (see also Wackett et al. [35]). Multicomponent monooxygenases are found in EC 1.14.13.-, 1.14.14.-, 1.14.15.-, and

TABLE 4. Substrate specificity of TSMOS and the products observed

Substrate	Relative activity ^a (%)	Identified product(s) ^b	Methods ^c
TS	100	4-Sulfobenzyl alcohol	CC and UV
<i>p</i> -Toluate	116	4-Carboxybenzyl alcohol	CC, UV, and MS
<i>m</i> -Toluate	38	Product not identified	
<i>o</i> -Toluate	6		
4-Ethylbenzoate	79	4-Carboxyphenyl ethanol ^d	UV and MS
<i>p</i> -Xylene	0		
Toluene	0		
Propane	5		
Terephthalate	0		
PSB	0		
4-Methoxybenzoate	54	4-Hydroxybenzoate HCHO	CC and UV Specific reaction
<i>p</i> -Cresol	10		
4-Carboxybenzyl alcohol	10	No transformation	
Benzoate	42	No transformation	
Benzenesulfonate	47	No transformation	
4-Hydroxybenzoate	10		
4-Hydroxybenzenesulfonate	0		
4-Aminobenzoate	0		
4-Aminobenzenesulfonate	54	No significant transformation	
4-Chlorobenzoate	12	No transformation	
4-Chlorobenzenesulfonate	28	No transformation	

^a Activity was measured as substrate-dependent oxygen uptake and standardized against TS as 100%.

^b Substrate disappearance and formation of products were determined by HPLC if the relative activity was >10%. Products were identified as indicated.

^c CC, cochromatography with authentic material (HPLC); UV, UV spectrometry; MS, mass spectrometry.

^d Data from UV spectrum: $\lambda_{\max}$, 197 and 236 nm; $\lambda_{\min}$, 214 nm. The PCI mass spectrum showed a protonated molecular ion at m/z 167 [(M + H)⁺], and the EI mass spectrum showed a peak at, e.g., m/z M - 45 (loss of hydroxylated side chain), consistent with either 1-(4-carboxyphenyl)-ethanol or 2-(4-carboxyphenyl)-ethanol.

1.14.99.-. Whichever is appropriate, we suggest that the recommended name be "4-toluene sulfonate monooxygenase system," which catalyzes the reaction "4-toluene sulfonate + NADH + H⁺ + O₂ = 4-sulfobenzyl alcohol + H₂O + NAD⁺." The systematic name would then be "4-toluene sulfonate:NADH: oxygen oxidoreductase (methyl-hydroxylating)," and the comments should be "iron-sulfur-flavoprotein (FMN) reductase, no independent ferredoxin, homomultimeric iron-sulfur oxygenase: requires iron." TSMOS bears little resemblance to the three-component alkane ω -monooxygenase of *Pseudomonas oleovorans*, whose oxygenase is membrane associated (25, 28); the three-component methane monooxygenase from *Methylosinus trichosporium* (13); or the microsomal cytochrome P-450-containing alkane monooxygenases (6).

A limited comparison is possible with the TOL plasmid-encoded xylene methyl-monooxygenase, apparently a two-component system (16), which has not been characterized biochemically but whose sequence (*xylA* and *xylM*) is now available (32). Xylene monooxygenase is believed to transform not only toluene but also its product benzyl alcohol, presumably to benzaldehyde. Hopper (18) also observed the formation of aldehyde by *p*-cresol-methylhydroxylase, whose primary product is the alcohol. TSMOS, however, did not significantly transform the alcohol tested (Table 4) and is active only on polar compounds. The M_r of the TOL-encoded xylene monooxygenase (41,500 for the presumed oxygenase [XylM] and 37,000 for the reductase [XylA], calculated from the nucleotide sequence), are similar to those of TSMOS. However, the components of TSMOS are soluble, whereas Suzuki et al. (32) anticipated a membrane-associated oxygenase component of the TOL-encoded enzyme analogous to that in the *P. oleovorans*

ω -monooxygenase (28). The amino acid compositions of the oxygenase components of TSMOS (Table 2), phthalate dioxygenase (2), and 4-methoxybenzoate *O*-demethylase (4) are similar, whereas the *xylM* gene product contains more hydrophobic amino acids (especially leucine and isoleucine), corresponding to its supposed location in the membrane. Since xylene monooxygenase is specific for apolar substrates, a location in the membrane would be understandable. TSMOS and the xylene monooxygenase system thus catalyze very similar chemical reactions and have similar M_r s but are presumably very different with respect to hydrophobicity.

Reductase B of TSMOS can transfer electrons from NADH to a number of electron acceptors, including the oxygenase component of PSBDOS, and we purified reductase B as the reductase of PSBDOS (22). It is known that some reductases can reduce several ferredoxins (31), and we were uncertain about the correct assignment of the reductases in *C. testosteroni* T-2 (22). The evidence presented above indicates that reductase B and oxygenase M are induced simultaneously and independently of the oxygenase component of PSBDOS. Assuming that the native oxygenase is a tetramer and is present at 4.3% of soluble protein and that the monomeric reductase (M_r , 36,000) represents 7.2% of soluble protein, the reductase is present in the cell in an eightfold excess over the native oxygenase. This idea is compatible with the effectively saturated oxygenase in crude extracts.

TSMOS and the 4-methoxybenzoate *O*-demethylase from *P. putida* (3) not only share similar structure and susceptibility to uncoupling, but each can also monooxygenate methyl aromatic groups and *O* demethylate. Whereas TSMOS shows a higher activity to hydroxylate *p*-toluate

than to demethylate 4-methoxybenzoate (Table 4), the enzyme from *P. putida* has the reverse specificity (3). It seems possible that the TSMOS in *C. testosteroni* T-2, which was isolated from enrichment cultures in TS-salts medium, did not evolve specifically to transform the xenobiotic sulfonated compound TS. Rather, it is an oxygenase with a broad substrate range for polar, *O*-methyl, and methyl aromatic compounds. Methoxy aromatic compounds are natural compounds which are widely distributed in nature as lignins (19). We suspect that TSMOS is a "natural" monooxygenase which was recruited, probably prior to the enrichment culture, into the degradative pathway of the sulfonated, xenobiotic substrate.

ACKNOWLEDGMENTS

We thank T. Vorherr and R. Falchetto, Biochemistry, ETH Zürich, Switzerland, for analyses of amino acid compositions and N-terminal amino acids and K. Ramsteiner, Ciba-Geigy AG, Basel, Switzerland, for MS analyses. We are grateful to S. Harayama for supplying a preprint (32).

This work was supported by a grant from the Kommission zur Förderung der Wissenschaftlichen Forschung (Project 1567).

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