Díaz, E., O'Laughlin, J. T., & O'Leary, M. H. (1988) *Biochemistry* (first of four papers in this issue).
Doherty, T. M., & Cleland, W. W. (1985) *Biochemistry 24*, 5875.
Fujita, N., Izui, K., Nishino, T., & Katsuki, H. (1984) *Biochemistry 23*, 1774.
Glattfeld, J. W. E., & Hoen, R. E. (1935) *J. Am. Chem. Soc. 57*, 1405.
Gonzalez, D. H., & Andreo, C. S. (1986) *Z. Naturforsch., C: Biosci. 41C*, 1004.
Gonzalez, D. H., Iglesias, A. A., & Andreo, C. S. (1986) *Arch. Biochem. Biophys. 245*, 179.
Lochmuller, H., Wood, H. G., & Davis, J. J. (1966) *J. Biol. Chem. 241*, 5678.
Maycock, A. L., Abeles, R. H., Salach, J. I., & Singer, T. P. (1976) *Biochemistry 15*, 114.
Mildvan, A. S., & Cohn, M. (1966) *J. Biol. Chem. 241*, 1178.
Muirhead, H., Clayden, D. A., Barford, D., Lorimer, C. G., Fothergill-Gilmore, L. A., Schiltz, F., & Schmitt, W. (1966) *EMBO J. 5*, 475.
Muller, E. (1964) *Methoden Org. Chem. (Houben-Weyl), 4th Ed. 12*, 275.
O'Leary, M. H. (1982) *Annu. Rev. Plant Physiol. 33*, 297.
O'Leary, M. H. (1983) *Physiol. Veg. 21*, 883.
Palm, R., Ohse, H., & Cherdron, H. (1966) *Angew. Chem., Int. Ed. Engl. 5*, 994.
Rambaud, R. (1934) *Bull. Soc. Chim. Fr.*, 1317.
Sauer, J. C. (1963) *Organic Syntheses*, Collect. Vol. IV, p 813, Wiley, New York.
Schwab, J. M., Ho, C.-K., Li, W., Townsend, C. A., & Salituro, G. M. (1986) *J. Am. Chem. Soc. 108*, 5309.
Sikkema, K. D., & O'Leary, M. H. (1988) *Biochemistry* (second of four papers this issue).
Stiborova, M., & Leblova, S. (1983) *Physiol. Veg. 21*, 1935.
Stiborova, M., & Leblova, S. (1986) *FEBS Lett. 205*, 32.
Williams, H. W. R., Cragoe, E. J., & Rooney, C. S. (1979) U.S. Patent 4 178 386.
Wirsching, P., & O'Leary, M. H. (1988) *Biochemistry* (third of four papers in this issue).

Benzylic Monooxygenation Catalyzed by Toluene Dioxygenase from *Pseudomonas putida*†

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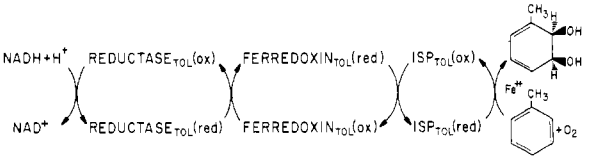
ABSTRACT: Toluene dioxygenase, a multicomponent enzyme system known to oxidize mononuclear aromatic hydrocarbons to *cis*-dihydrodiols, oxidized indene and indan to 1-indenol and 1-indanol, respectively. In addition, the enzyme catalyzed dioxygen addition to the nonaromatic double bond of indene to form *cis*-1,2-indandiol. The oxygen atoms in 1-indenol and *cis*-1,2-indandiol were shown to be derived from molecular oxygen, whereas 70% of the oxygen in 1-indanol was derived from water. All of the isolated products were optically active as demonstrated by ^{19}F NMR and HPLC discrimination of diastereomeric esters and by chiroptic methods. The high optical purity of (−)-(1*R*)-indanol (84% enantiomeric excess) and the failure of scavengers of reactive oxygen species to inhibit the monooxygenation reaction supported the contention that the monooxygen insertion is mediated by an active-site process. Experiments with 3-[^2H]indene indicated that equilibration between C-1 and C-3 occurred prior to the formation of the carbon–oxygen bond to yield 1-indenol. Naphthalene dioxygenase also oxidized indan to 1-indanol, which suggested that benzylic monoxygenation may be typical of this group of dioxygenases.

Oxygenases are enzymes that incorporate molecular oxygen into organic compounds (Hayaishi et al., 1955). The importance of these enzymes in mammalian and microbial metabolism has been well documented (Hayaishi, 1982; Dagley, 1986).

In the case of unsubstituted aromatic hydrocarbons, different reaction mechanisms are used by mammals and bacteria to initiate oxygenation of the benzenoid nucleus. For example, mammals utilize a monooxygenase enzyme system to incorporate one atom of molecular oxygen into the aromatic ring. The initial reaction products are arene oxides that can react with cellular nucleophiles, isomerize to form phenols, or undergo enzymatic hydration to form *trans*-dihydrodiols (Daly et al., 1972). In contrast, bacteria utilize a dioxygenase enzyme system to initiate the oxidation of the aromatic nucleus. Both atoms of molecular oxygen are incorporated into the substrate, and the first detectable products are dihydrodiols in which the hydroxyl groups have a cis-relative stereochemistry (Gibson & Subramanian, 1984).

Scheme I



Toluene dioxygenase oxidizes toluene to (+)-(1*S*,2*R*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol; Gibson et al., 1970; Kobal et al., 1973; Ziffer et al., 1973).

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This reaction is catalyzed by a multicomponent enzyme system that has been isolated from toluene-grown cells of *Pseudomonas putida*. The organization of the individual components of toluene dioxygenase is shown in Scheme I.

Electrons are transferred from NADH[1] through a flavoprotein (reductase$_{TOL}$) and a [2Fe-2S] protein (ferredoxin$_{TOL}$) to a terminal iron–sulfur protein (ISP$_{TOL}$) that catalyzes the oxidation of toluene to *cis*-toluene dihydrodiol. Although these individual components have been characterized in some detail (Subramanian et al., 1981, 1985; Gibson et al., 1982), little is known about the oxygen insertion reaction.

The observation that toluene dioxygenase will oxidize indole to indigo (Ensley et al., 1983) shows that this enzyme can oxidize indole to indoxyl or some unstable intermediate that can generate indoxyl. A logical extension of these results indicated that indene and indan may also serve as substrates for toluene dioxygenase and provide useful information on the mechanism of the oxygenation reaction.

We now report that toluene dioxygenase can catalyze the monooxygenation of indene and indan to benzylic alcohols. The mechanism of the monooxygenation reaction has been probed by stereochemical and isotope-labeling experiments. In addition, the observation that naphthalene dioxygenase also forms 1-indanol from indan suggests that benzylic monooxygenation may be a characteristic of bacterial multicomponent dioxygenases.

Materials and Methods

Analytical Methods. High-performance liquid chromatography (HPLC) was performed with a Waters Associates Model 6000A solvent delivery system with a U-6K septumless injector and a Model 440 absorbance detector operated at 254 nm. Peak integrations were determined with a Spectra Physics Model SP4270 integrator. Experimental conditions used for separations are described in the text. NMR spectra were recorded on a Varian Model EM-390 spectrometer, a Brucker Model WHI-90 spectrometer, or a Nicolet Model NT-200 spectrometer. Low-resolution mass spectra were obtained with a Finnegan Model 4023 spectrometer. Absorption spectra were obtained with an Aminco DW-2 spectrophotometer and a Beckman Model 25 spectrophotometer. Optical rotations were determined with a Perkin-Elmer 241 MC polarimeter.

Materials. Indene 1,2-oxide was prepared by reacting indene with *m*-chloroperoxybenzoic acid in methylene chloride and also by the method of Imuta and Ziffer (1978). Hydration of the oxirane ring with 3% perchloric acid in *tert*-butyl alcohol gave a mixture of *cis*- and *trans*-1,2-indandiol, which were separated and purified by silica gel column chromatography. The relative stereochemistry of the hydroxyl groups in each diol was determined by NMR spectrometry (Imuta & Ziffer, 1978). Reaction of *cis*-1,2-indandiol with 2,2-dimethoxypropane gave an acetonide derivative whose NMR spectrum (90 MHz, $CDCl_3$) showed bands at δ 1.1 (s, 3 H), 1.3 (s, 3 H), 3.0 (d, 2 H), 4.8 (m, 1 H), 5.4 (d, 1 H), and 7.0–7.4 (m, 4 H). 1-Indenol was prepared by reacting indene with a 30% molar excess of *n*-butyllithium in tetrahydrofuran. The reaction was conducted for 15 min under an argon atmosphere at −78 °C. The resulting anion was quenched with oxygen to yield the hydroperoxide that was reduced with acidic potassium iodide to yield 1-indenol. [3-^{2}H]Indene was prepared by reduction of 1-indanone with lithium aluminum deuteride followed by acidification to eliminate water from [1-^{2}H]-indanol. The reaction product, [3-^{2}H]indene, was distilled under vacuum and gave an NMR spectrum (90 MHz) that showed bands at δ 3.0 (d, 2 H), 6.2 (s, 1 H), and 6.9–7.3 (m, 4 H). A small signal at δ 6.6 indicated that the product was at least 97% pure. 1-Indanone semicarbazone was synthesized by reacting 1-indanone with excess semicarbazide hydrochloride in 95% ethanol. 2-Indanone was prepared as previously described (Horan & Schiessler, 1973).

Table I: Properties of Bacterial Strains

bacterium	relevant characteristics
P. putida F1	wild type; source of toluene dioxygenase enzyme components (Gibson et al., 1968)
P. putida F39/D	derivative of PpF1; accumulates products of toluene dioxygenase (Gibson et al., 1970)
P. putida F106	derivative of PpF1 (Finette et al., 1984)
Pseudomonas sp. NCIB 9816	wild type; source of naphthalene dioxygenase enzyme components (Davies & Evans, 1964)
Pseudomonas sp. NCIB 9816-11	lacks active dihydrodiol dehydrogenase; accumulates *cis*-naphthalene dihydrodiol (Gibson, unpublished results)
Pseudomonas sp. NICB 9816-C2	strain cured of plasmid encoding naphthalene dioxygenase (C. Serdar, unpublished data)

The following chemicals were obtained from Aldrich Chemical Co., Milwaukee, WI: indene (99%), indan, 1-indanol, 2-indanol, 5-indanol, 1-indanone, (−)-α-methoxy-α-[4-(trifluoromethyl)phenyl]acetic acid, and (−)-menthoxyacetic acid. NADH and NAD$^+$ were from Sigma Chemical Co., St. Louis, MO. Hydrazine was obtained from Eastman Chemical Co., Rochester, NY. Oxygen-18 (98% isotopic purity) was purchased from Stauffer Chemical Co., Richmond, CA, and oxygen-18 water (20.6 atom %) was from Pro-Chem, Oxford, NJ. All other reagents used were of the highest purity commercially available.

Growth of Pseudomonas Strains. Bacterial strains used in the present investigation are listed in Table I. *Pseudomonas putida* F1 and its derivatives were grown on a mineral salts medium as described previously (Finette et al., 1984). Hydrocarbon substrates (toluene, indene, and indan) were added in the vapor phase as described previously for ethylbenzene (Gibson et al., 1973). Strains were preinduced for toluene dioxygenase activity by growing cells in the presence of toluene to a turbidity at 600 nm of 1.3–1.6. Cells were harvested by centrifugation, resuspended in fresh growth media (100 mL or 2.5 L), and incubated with the appropriate transformation substrates. Each transformation was conducted for 1 h.

Pseudomonas sp. NCIB 9816-11 was grown in an identical manner as described for strain F1 except that 0.35 mM anthranilic acid was used in place of toluene to induce naphthalene dioxygenase. After the cells were harvested and resuspended in fresh growth medium, the transformation with indan was conducted for 30 min.

Isolation and Purification of Metabolites Formed from Indene by P. putida F39/D. A 2.5-L culture of *P. putida* F39/D was incuated for 1 h in the presence of indene. Ethyl acetate extraction of the culture filtrate followed by conventional workup gave 300 mg of a solid residue that was analyzed by HPLC and thin-layer chromatography (TLC). Three

[1] Abbreviations: HPLC, high-pressure liquid chromatography; MTPA, (−)-α-methoxy-α-[4-(trifluoromethyl)phenyl]acetic acid; MTPA-Cl, (−)-α-methoxy-α-[4-(trifluoromethyl)phenyl]acetyl chloride; MOA, (−)-menthoxyacetic acid; MOA-Cl, (−)-menthoxyacetyl chloride; NADH, reduced nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SOD, superoxide dismutase.

products (compounds I, II, and III) were detected by each analytical procedure.

Silica gel column chromatography was used to isolate compounds I, II, and III that gave the following physical properties. Compound I (106 mg): mp 95–96 °C; λ_{max}^{MeOH} (ϵ, cm^{-1} M^{-1}) 259 (514), 265 (781), and 272 (836) nm; NMR (90 MHz, pyridine-d_5) δ 3.1–3.2 (dd, 2 H), 4.6 (d, 1 H), $J_{2,3}$ = 5.1 Hz, 5.0 (bs, 2 H), 5.2 (d, 1 H), $J_{3,2}$ = 5.1 Hz, 7.1–7.7 (m, 4 H); mass spectrum, m/z M^+ 150 (15), $M^+ - H_2O$, 132 (44), and 104 (100); $[\alpha]^{25}_D$ −14.6° (c 5.5, $CHCl_3$). Compound II (117 mg): mp 54–56 °C; λ_{max}^{MeOH} (ϵ, cm^{-1} M^{-1}) 265 (4300), 290 (920), 229 (440) nm; NMR (90 MHz, $CDCl_3$) δ 2.50 (br s, 1 H), 5.0 (s, 1 H), 6.2 (dd, 1 H), 6.6 (1 H), and 7.0–7.4 (m, 4 H); mass spectrum, m/z M^+ 132 (100); $[\alpha]^{25}_D$ +87.2° (c 0.3, $CHCl_3$). Compound III (2 mg) showed absorption maxima in MeOH at 244, 286, and 290 nm. Reaction of compound III with semicarbazide hydrochloride gave a white crystalline product with the following properties: mp 231–236 °C; λ_{max}^{MeOH} 268, 278, 297, and 308 nm; mass spectrum m/z, M^+ 189 (43).

Isolation and Identification of Metabolites Formed from Indan by P. putida F39/D. *P. putida* F39/D was incubated with indan as described above for indene. The residue obtained after ethyl acetate extraction was analyzed by TLC and showed the presence of a major unidentified product (compound IV) and a small amount of *cis*-toluene dihydrodiol. The latter compound presumably was carried over from the preincubation stage when toluene was used to induce the dioxygenase enzyme system. Silica gel chromatography gave 116 mg of compound IV as a white crystalline solid with the following physical properties: mp 51–53 °C; λ_{max}^{MeOH} (ϵ, cm^{-1} M^{-1}) 259 (590), 265 (905), and 272 (980) nm; NMR (200 MHz, $CDCl_3$) 1.9 (bs, 1 H), 1.9–2.6 (m, 2 H), 2.7–3.1 (m, 2 H), 5.2 (t, 1 H), and 7.2–7.5 (m, 4 H); mass spectrum, m/z M^+ 134 (60), $M^+ - H_2O$, 116 (100); $[\alpha]^{25}_D$ −23.4° (c 4.8, $CHCl_3$).

Analysis of Chiral Products. Optical rotations of metabolites were determined under the same conditions described in the literature. Values used were $[\alpha]^{25}_D$ −30.8° for (1*R*)-indanol (Boyd et al., 1982) and $[\alpha]^{25}_D$ −51.0° for (1*S*,2*R*)-*cis*-1,2-indandiol (Imuta & Ziffer, 1978).

Diastereomeric esters were prepared with (−)-menthoxyacetyl chloride (MOA-Cl) or (−)-α-methoxy-α-[4-(trifluoromethyl)phenyl]acetyl chloride (MTPA-Cl) as chiral reagents (Dale et al., 1969). Diastereomers were separated by reverse-phase HPLC (1-indanyl-MTPA) on a Beckman Ultrasphere 5 μm C-18 column (solvent, MeOH–H_2O, 75:25; flow rate 1.3 mL/min) and by normal-phase HPLC (*cis*-1,2-indandiol–MOA) on two consecutive Waters Porasil semipreparative columns (solvent, ethyl acetate–hexane, 3:97; flow rate 1.1 mL/min). Under these conditions base-line resolution of diastereomers was obtained and peak areas were determined by integration.

1-Indanyl-MTPA diastereomers were also analyzed by ^{19}F NMR. Reaction products were dissolved in $CDCl_3$ and spectra recorded on a Varian EM-390 or a Bruker WHI-90 FT-NMR spectrometer. Chemical shifts were determined by using $CFCl_3$ as an external standard.

It was not possible to determine the enantiomeric composition of 1-indenol directly due to the facile isomerization of this metabolite to 1-indanone under conditions used for the synthesis of diastereomeric esters. Consequently, 1-indenol was reduced to 1-indanol prior to derivatization. 1-Indenol (1.26 mg) was dissolved in 0.60 mL of methanol followed by 2.5 μL of glacial acetic acid, several crystals of $CuCl_2 \cdot 2H_2O$, and 10 μL of hydrazine. The reaction was conducted at room temperature and the reaction monitored by HPLC. After 24 h, the reaction mixture was applied to the top of a small column (3.0 × 0.5 cm) of 20% deactivated silica gel. 1-Indanol was eluted with methylene chloride and derivatized with MTPA-Cl as described above.

The identities of the diastereomers formed from hydroxylated indan and indene metabolites were confirmed by comparison of their HPLC retention times, ultraviolet spectra, and mass spectra with those of diastereomers formed from racemic 1-indanol and *cis*-1,2-indandiol.

Incorporation Studies with Oxygen-18 and Oxygen-18 Water. Toluene-induced cells (45-mL culture) of *P. putida* F39/D were placed in 65-mL serum bottles that were sealed with a rubber septum. The atmosphere above the cells was replaced with argon by alternately evacuating and purging each serum bottle with argon gas. After four cycles, 20 mL of argon was removed with a Precision gastight syringe and replaced with 20 mL of $^{18}O_2$ (indan oxidation) or 10 mL each of $^{18}O_2$ and $^{16}O_2$ (indene oxidation). The oxygen composition of the gas in the headspace above the reaction mixtures was determined by mass spectrometry. Analyses were performed at the beginning and end of each experiment. Reactions were initiated by the addition of indan or indene to a final concentration of 1.6 mM. After 30 min, cells were removed by centrifugation and the supernatant solutions extracted with 2 volumes of ethyl acetate. The solvent was removed in vacuo and each residue dissolved in a minimal volume of methylene chloride. Reaction products were isolated by HPLC on a Beckman Ultrasphere 5-μm silica column. 1-Indanol and 1-indenol were eluted with methylene chloride at a flow rate of 3.0 mL/min. *cis*-1,2-Indandiol was eluted with a 6-min linear gradient of 2-propanol in methylene chloride. The final concentration of 2-propanol was 15%. The oxygen composition of each purified metabolite was determined by mass spectrometry.

Indan oxidation by *P. putida* F39/D was also determined in the presence of oxygen-18 water. The experimental conditions were the same as described above with the exception that a normal air phase was present and the cells were suspended in 20.6 atom % oxygen-18 water and allowed to equilibrate without agitation for 15 min prior to addition of indan to a final concentration of 3.0 mM. It was shown previously that equilibration between intracellular and extracellular water occurs very rapidly, less than 1 min being required for complete equilibration (Spencer et al., 1980). Mass spectral analysis of the reaction medium indicated that the water was 18.7% enriched in oxygen-18. The 1-indanol formed was isolated as described above and the oxygen-18 content determined by mass spectrometry.

Toluene Dioxygenase and Naphthalene Dioxygenase. The three components of the toluene dioxygenase enzyme system were purified as previously described (Subramanian et al., 1979, 1981). These were ferredoxin$_{TOL}$ reductase, ferredoxin$_{TOL}$, and the terminal oxygenase (ISP$_{TOL}$). The first two enzymes gave single bands that stained for protein when analyzed on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate. ISP$_{TOL}$ is an unstable protein, and the sample used in the experiments described below was approximately 50% pure. Naphthalene dioxygenase was a kind gift from Dr. Billy Haigler of the University of Texas at Austin. The enzyme had been purified as described previously (Ensley et al., 1982; Ensley & Gibson, 1983).

Oxidation of Indan by Purified Toluene Dioxygenase. Reaction mixtures were 5 mL in volume and contained 0.05

M Tris-HCl, pH 7.5; ferredoxin$_{TOL}$ reductase, 15 μg of protein; ferredoxin$_{TOL}$, 65 μg of protein; ISP$_{TOL}$, 1.57 mg of protein; ferrous sulfate, 0.36 μM; indan, 0.84 mM; and NADH, 0.6 mM. The order of addition of each component was as indicated. After 10 min at 30 °C, reaction mixtures were extracted with ethyl acetate and analyzed by HPLC.

Indan Oxidation by Purified Naphthalene Dioxygenase. Naphthalene dioxygenase components were incubated with substrates in 1.0 mL of the same buffer system as for toluene dioxygenase. In addition, reaction mixtures contained 20 μM FAD, 0.5 mM NADH, 1% (v/v) *N,N*-dimethylformamide, and 1 mM indan. After 15 min at 30 °C, reaction mixtures were extracted with ethyl acetate and analyzed by HPLC.

RESULTS

Oxidation of Indene and Indan by P. putida F39/D. Three products were formed from indene oxidation by *P. putida* F39/D. Compound I was identified as a dihydroxyindan derivative from its NMR and mass spectral properties (see Materials and Methods). It did not readily undergo acid-catalyzed dehydration. Elimination of water under mild conditions to form phenols is typical of *cis*-dihydrodiols that are formed by oxidation of the aromatic nucleus. However, compound I did form an acetonide derivative with 2,3-dimethoxypropane. These observations suggested that compound I was *cis*-1,2-indandiol, and this structural assignment was confirmed by showing that compound I and synthetic *cis*-1,2-indandiol had identical physical (NMR, mass spectrum, HPLC) properties. Compounds II and III had physical (NMR, mass spectrum, HPLC) properties identical with those given by synthetic 1-indenol and 1-indanone, respectively. The yields of isolated metabolites from 300 mg of reaction products were *cis*-1,2-indandiol (35%), 1-indenol (39%), and 1-indanone (1%). The stability of *cis*-1,2-indandiol makes it unlikely that the monooxygenated products are derived from this metabolite, and in this context, it is important to note that when heated in strong acid, *cis*-1,2-indandiol eliminates water to yield 2-indanone.

A single major product was formed from indan by *P. putida* F39/D. This compound had physical (NMR, mass spectrum, HPLC) properties identical with those given by synthetic 1-indanol. In a parallel experiment with strain *P. putida* F106, a mutant that lacks toluene dioxygenase activity, no discernible products were observed.

With the exception of optical rotation, the hydroxylated metabolites were shown to be identical with known synthetic compounds. HPLC analyses of crude reaction products failed to show the formation of *trans*-1,2-indandiol, 2-indanol, 5-indanol, 2-indanone, and 1,2-indene oxide from either indene or indan. In addition, there were no detectable amounts of metabolites that would have resulted from oxidation of the aromatic nucleus of either substrate.

When *P. putida* F39/D was incubated with either indene or indan for periods longer than 1 h, significant amounts of 1-indanone were formed from each substrate. The formation of 1-indanone from indene led to a decrease in the observed ratio of 1-indenol to *cis*-1,2-indandiol. This result, together with reported data on the isomerization reaction of 1-indenol to 1-indanone (Friedrich & Taggert, 1975), suggests that 1-indenol is the direct precursor of the ketone detected in the microbial transformation of indene. In indan transformations, 1-indanone was shown to be derived from 1-indanol by the action of a toluene-inducible 1-indanol dehydrogenase. The physiological significance of this activity is unknown, but, pertinent to this investigation, experiments indicated that the dehydrogenase did not significantly alter the stereochemical integrity of products isolated from culture fluids. In all experiments less than 2% of the total products was 1-indanone. Furthermore, 1-indanone was not reduced to 1-indanol by whole cells or crude extracts containing NADH and NADPH.

Table II: Indan Monooxygenase Activity of Toluene Dioxygenase

reaction mixture	activity (nmol/min)
complete[a]	5.6 ± 1.6
complete − reductase$_{TOL}$, ferredoxin$_{TOL}$, ISP$_{TOL}$	<0.1
complete − reductase$_{TOL}$, ferredoxin$_{TOL}$	<0.1
complete − ISP$_{TOL}$	<0.1
complete − NADH	<0.1
complete under argon	<0.1
complete − Fe^{2+}	1.1 ± 0.4
complete + catalase[b]	6.2 ± 1.2
complete + SOD[b]	5.0 ± 1.1
complete + catalase + SOD	5.8 ± 2.0
complete + 40 mM mannitol	6.9 ± 2.0
complete + 2 mM L-tryptophan	5.1 ± 1.8
complete + 10 mM L-histidine	4.9 ± 1.6

[a] For complete reaction mixture contents, see Materials and Methods. [b] Concentration was 100 units/mL.

Monooxygenation Reactions Catalyzed by Purified Toluene Dioxygenase. Purified oxygenase components were used in vitro in an effort to investigate the ability of the enzyme to produce monooxygenated products, to confirm that 1-indanone was not a primary product of toluene dioxygenase, and to examine characteristics of the reaction that would not be possible in in vivo experiments. First, both 1-indenol and 1-indanol were products derived from the oxidation of indene and indan, respectively, by toluene dioxygenase. Second, 1-indanone was not observed in 15-min enzyme incubations with either substrate but was observed only on longer incubations with indene. This observation is consistent with 1-indanone being derived from 1-indenol by an isomerization reaction. The *cis*-diol derived from indene was obscured by polar materials that were also present in zero-time reaction mixtures. Thus, the monooxygenation reaction was further investigated by monitoring indan oxidation, as this gave a single product that could be readily quantified.

1-Indanol formation was observed to be linear for at least 15 min (Table II). Product was not detected when one or more of the three components or the enzyme system were absent, when NADH was omitted, or when argon replaced air in the gas phase over the reaction. The deletion of Fe^{2+} from the reaction mixture resulted in an 80% diminution in the amount of 1-indanol formed. A similar decrease (of 75%) was observed in *cis*-toluene dihydrodiol formation when iron was not included in the assay with toluene. Catalase and superoxide dismutase, either singly or in combination, did not inhibit indan monooxygenation or toluene dihydrodiol formation. Furthermore, the hydroxyl radical scavengers mannitol and L-tryptophan and the singlet oxygen scavenger L-histidine did not decrease the amount of 1-indanol formed by toluene dioxygenase. These observations suggest that 1-indanol and 1-indenol are formed at the active site of the enzyme. If this is the case, oxygen insertion should proceed with a high degree of stereochemical control since a number of aromatic *cis*-dihydrodiols formed by this enzyme have been shown to be single enantiomers (Gibson, 1984).

Absolute Stereochemistry of Hydroxylated Products Formed from Indene and Indan by P. putida F39/D. The dihydroxyindan formed from indene was shown to be (−)-*cis*-(1*S*,2*R*)-dihydroxyindan, which was obtained in 28% enantiomeric excess. This conclusion was based on a specific rotation of −51.0° determined for the optically pure (−)-en-

Table III: Absolute Stereochemistry of Hydroxylated Products

enzyme	substrate	product	% enantiomeric excess as determined by optical rotation	% enantiomeric excess as determined by HPLC[a]
toluene dioxygenase	indene	(−)-*cis*-(1*S*,2*R*)-dihydroxyindan	28[b]	32
		(+)-(1*S*)-indenol	*c*	26[d]
	indan	(−)-(1*R*)-indanol	76[e]	84
naphthalene dioxygenase	indan	(+)-(1*S*)-indanol	100	92

[a] Determined by integration of peak areas following HPLC resolution of diastereomeric acids as described under Materials and Methods. [b] Based on rotational data from Imuta and Ziffer (1978). [c] Rotational data not available for 1-indenol. [d] Determined following reduction to 1-indanol as described in the text. [e] Based on rotational data from Boyd et al. (1982).

antiomer (Imuta & Ziffer, 1978). Further confirmation was provided by reacting the bacterial metabolite with (−)-MOA-Cl and separation of the resulting diastereomers by HPLC (Materials and Methods). A ratio of 66:34 was obtained for the two diastereomers, indicating a 32% enantiomeric excess of (−)-*cis*-(1*S*,2*R*)-dihydroxyindan (Table III).

Several different specific rotation values have been reported for 1-indanol. The value of +30.8° reported for (+)-(1*S*)-indanol (Boyd et al., 1982) was used to calculate an enantiomeric excess of 76% for the (−)-(1*R*)-indanol formed from indan by toluene dioxygenase. This value was confirmed by ^{19}F NMR analysis of the diastereomers formed by reacting the metabolite with (−)-MTPA-Cl. The results show different chemical shifts (δ 71.76 and 71.93) corresponding to CF_3 in the two diastereomers. Integration of peak areas gave a ratios of 9:1, indicating an 80% enantiomeric excess of (−)-(1*R*)-indanol. A control experiment with diasteromers prepared from synthetic 1-indanol gave a peak ratio of 1:1. Similar results were obtained when the diasteromers were separated by HPLC (Table III).

Determination of the enantiomeric composition of 1-indenol formed from indene by toluene dioxygenase was complicated by the instability of the alcohol under alkaline conditions. Attempts to form diastereomeric esters with chiral MOA or MTPA chlorides in the presence of pyridine or triethylamine resulted in isomerization to 1-indanone. Similar results were obtained under milder conditions with MTPA-imidazole. The enantiomers were not resolved chromatographically on a Pirkle (chiral) HPLC column and could not be distinguished by NMR analysis in the presence of the chiral shift reagent tris[3-[(trifluoromethyl)hydroxymethylene]-*d*-camphorato]-europium. Consequently, 1-indenol was reduced to 1-indanol with diimide reagent. The 1-indanol was derivatized with (−)-MTPA-Cl, and the resulting diasteromers were separated by HPLC as described above. The major diastereomer formed was of opposite configuration to that observed with 1-indanol formed from indan by toluene dioxygenase. Integration of

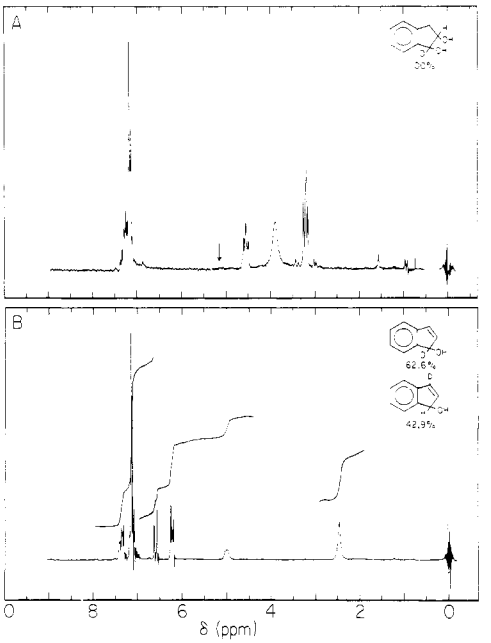


FIGURE 1: Proton NMR spectra of (A) *cis*-1,2-indandiol and (B) 1-indenol isolated from a culture filtrate of *P. putida* F39/D incubated with [3-^{2}H]indene as described under Materials and Methods. The arrow marks the position of the proton on C-1 of synthetic *cis*-1,2-indandiol. The products depicted by each spectrum are shown in the upper right corners. The assignments in spectrum B are δ 2.5 (CH-O*H*), 5.0 (C*H*OH), 6.2 (C*H*CHOH), 6.6 (C*H*Ar). Integrations were normalized to the protons on C-2 and the aromatic ring, and the values expressed are the averages of five recorded spectra.

peak areas showed that indene is oxidized to (+)-(1*S*)-indenol, which is formed in 26% enantiomeric excess.

Oxidation of [3-^{2}H]Indene by P. putida F39/D. 1-Indenol and *cis*-1,2-indandiol formed from [3-^{2}H]indene by *P. putida* F39/D were isolated as described above. The position of the deuterium in each metabolite was determined by NMR analysis, and the results are shown in Figure 1. All of the deuterium was located at the C-1 position in *cis*-1,2-indandiol, indicating that double-bond isomerization did not occur prior to oxygen insertion. In contrast, the isolated 1-indenol contained deuterium at both the C-1 and C-3 positions. These results indicated that 1-indenol formation is preceded by the formation of an intermediate state in which there is equilibration between the C-1 and C-3 positions.

Oxidation of Indene and Indan in the Presence of Oxygen-18 or Oxygen-18 Water. Indene was incubated with *P. putida* F39/D in the presence of an atmosphere containing $^{18}O_2$ and $^{16}O_2$ (59:41). 1-Indenol and *cis*-1,2-indandiol were isolated by HPLC and analyzed by mass spectrometry. The results (Table IV) show that the hydroxyl groups in *cis*-1,2-indandiol are derived from a single molecule of atmospheric oxygen and that one atom of molecular oxygen is incorporated into 1-indenol. In similar experiments with indan, the amount of oxygen incorporated into 1-indanol was found to be 30%

Table IV: Incorporation of Oxygen-18 into Indene and Indan by Toluene Dioxygenase

source of oxygen-18	substrate	product	isotope enrichment of oxygen source (%)	product oxygen-18 enrichment[a] (%)	normalized enrichment[b] (%)
$^{18}O_2$	indene	1-indenol	59	54	92
$^{18}O_2$	indene	*cis*-1,2-indandiol	59	50	85
$^{18}O_2$	indan	1-indanol	90	26	29
			75	23	31
$[^{18}O]H_2O$	indan	1-indanol	18.7	12.7	68

[a] This was determined by (M + 2)/[M + (M + 2)] × 100 for 1-indenol and 1-indanol and (M + 4)/[M + (M + 4)] × 100 for *cis*-1,2-indandiol. [b] Calculated from product oxygen-18 enrichment/isotope enrichment of oxygen source × 100.

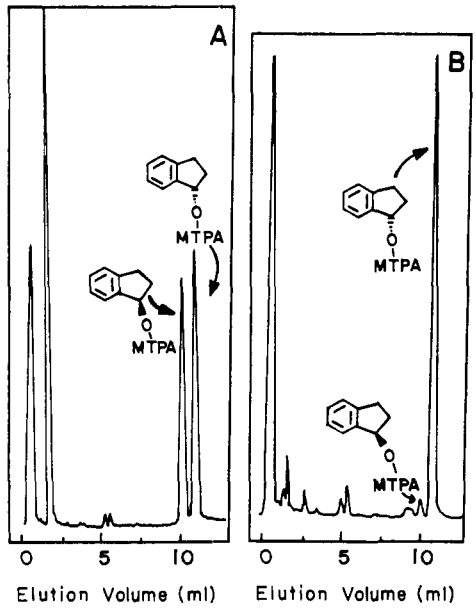


FIGURE 2: HPLC resolution of 1-indanyl-MTPA diastereomers derived from the reaction of (−)-(*R*)-MTPA-Cl with (A) synthetic 1-indanol and (B) 1-indanol isolated from a culture filtrate of *Pseudomonas* sp. NCIB 9816-11 as described under Materials and Methods. HPLC was conducted with a Beckman ultrasphere 5 μm C18 column eluted isocratically with 80% methanol in water at a flow rate of 1 mL/min.

(Table IV). The optical purity of the 1-indanol formed in this experiment was determined as described above, and the enantiomeric excess of (−)-(1*R*)-indanol was shown to be 88%, confirming that it was formed enzymatically.

Indan oxidation by *P. putida* F39/D was also conducted in the presence of $[^{18}O]H_2O$ (18.7 atom %). As shown in Table IV, 68% of the oxygen in 1-indanol was derived from water.

Oxidation of Indan by Naphthalene Dioxygenase. Pseudomonas sp. NCIB 9816-11 oxidized indan to form 1-indanol. A derivative strain, *Pseudomonas* sp. NCIB 9816-C2, that does not contain the genes for naphthalene dioxygenase did not produce detectable products from indan. Further experiments with purified naphthalene dioxygenase demonstrated that this was the enzyme responsible for the formation of 1-indanol. The absolute stereochemistry of the alcohol was examined by using 1-indanol purified from culture filtrates of *Pseudomonas* sp. NCIB 9816-11. The specific rotation was +32°, indicating that the alcohol was of high optical purity and had the opposite sign of rotation when compared to 1-indanol formed by toluene dioxygenase (Table III). The identity of the product as (+)-(1*S*)-indanol was confirmed by preparing diastereomeric esters that were separated by HPLC as shown in Figure 2.

Discussion

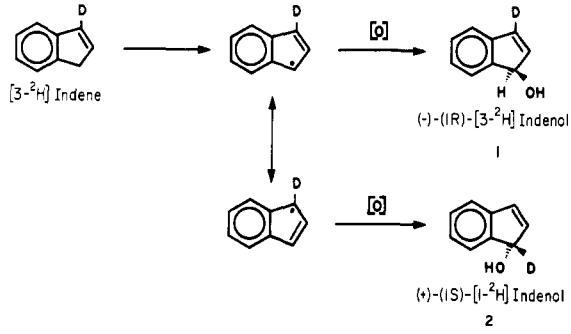
The results obtained from studies with mutant strains and with purified enzyme components demonstrated that toluene dioxygenase and naphthalene dioxygenase can catalyze monooxygenation reactions when provided with appropriate substrates. It is of interest that another enzyme from *Pseudomonas*, 4-methoxybenzoate *monooxygenase*, has been shown to dihydroxylate the vinylic side chain of 4-methoxystyrene with both atoms derived from molecular oxygen (Wende et al., 1982). The oxygen-activating component of 4-methoxybenzoate monooxygenase contains 2Fe-2S centers and additional non-heme iron in a 1:1 stoichiometry (Bernhardt et al., 1982). Similarly, the redox active groups of toluene dioxygenase are comprised of two 2Fe-2S clusters and two additional ferrous iron when fully activated (Subramanian and Gibson, unpublished observations). Furthermore, the 2Fe-2S clusters of both enzymes may be of the Rieske-type, containing two cysteine and two non-cysteine ligands (Fee et al., 1984). The structural similarities and the overlap in reactivities observed for toluene dioxygenase and 4-methoxybenzoate monooxygenase show that mechanistic distinctions between dioxygenase and monooxygenase activities should be treated with caution. Both oxygenases are still somewhat ill-defined with respect to the coordination of their mononuclear iron centers and the means by which they insert dioxygen into aromatic substrates. Consequently, the monooxygenation reactions may be extremely useful for dissecting the catalytic pathway. In another example, the heme enzyme indoleamine 2,3-dioxygenase has been shown to catalyze oxidative demethylation and aromatic ring monooxygenation. The study of these reactions has provided important mechanistic insights (Takikawa et al., 1983).

It is worth comparing the characteristics of the benzylic monooxygenation reaction described here with similar reactions catalyzed by the most well-studied monooxygenase, cytochrome P-450. Oxygen-18 experiments with toluene dioxygenase indicated that molecular oxygen is being activated and attacks the benzylic carbon atom. Although only 30% of the oxygen in enzymatically formed 1-indanol is derived from molecular oxygen, oxygen activation by toluene dioxygenase is essential for catalysis as indicated by (i) the failure to observe products in the absence of O_2, (ii) the requirement for NADH to catalyze monooxygenation, and (iii) the comparable stimulation of monooxygenation and dioxygenation reactions by addition of ferrous iron to reaction mixtures. An iron-bound oxygen species may be the site of exchange of oxygen with water. This type of exchange has also been proposed to explain the incorporation of oxygen from water in substrate hydroxylation by hepatic cytochrome P-450 (Nordbloom et al., 1976; MacDonald et al., 1982) and cytochrome $P\text{-}450_{CAM}$ (Heimbrook & Sligar, 1981) with cumene hydroperoxide and iodosobenzene as oxygen donors. More recently, resonance Raman spectroscopic studies have provided direct evidence for the exchange of the oxygen atom in the ferryl oxene form (Fe^{IV}=O) of horseradish peroxidase with water at pH 7.0 (Hashimoto et al., 1986).

Further support for an active-site mechanism in the monooxygenation reaction catalyzed by toluene dioxygenase comes from the observed high degree of stereochemical control in monohydroxylation and the failure of scavengers of active oxygen species to inhibit the reaction. It is interesting that toluene dioxygenase and naphthalene dioxygenase form the opposite enantiomers of 1-indanol, each in high optical yield, reflective of differences in substrate alignment in the active site. In contrast, hepatic cytochrome P-450 shows less stereochemical control as evidenced by the isolation of (+)-(1*S*)-indanol in 24–52% enantiomeric excess from microsomal incubations with indan (Billings et al., 1970).

The difference in absolute configuration between the alcohols obtained from indan and indene is understandable when considered in concert with the results of the experiment conducted with [3-^{2}H]indene. It was shown that the deuterium label is found specifically at C-1 in *cis*-1,2-indandiol (Figure 1), which demonstrates that deuterium scrambling did not occur before or during substrate dioxygenation. However, in 1-indenol the deuterium is partitioned between C-1 and C-3 with 1-[1-^{2}H]indenol in 20% excess with respect to 1-[3-^{2}H]indenol. The scrambling of deuterium would be consistent with an equilibrating intermediate, shown in Scheme II as a carbon-centered radical, that could be quenched with an ox-

Scheme II



ygen atom from the top face to give either of the deuteriated products and the respective absolute configuration as shown. Note that compound **2** in Scheme II is the preferred deuteriated product in 20% excess, and furthermore, it represents the preferred enantiomer found in 26% excess. These data do not directly demonstrate that a carbon-centered radical intermediate is the equilibrating species, although similar intermediates have been implicated in hydroxylations catalyzed by cytochrome P-450 monooxygenases (Gelb et al., 1982; Groves et al., 1978; White et al., 1986) and by a non-heme iron α-ketogluturate-dependent dioxygenase (Englard et al., 1985). In this regard, future use of 1,1-dideuterioindan as a substrate for toluene dioxygenase may prove useful as a probe for a primary isotope effect in the monooxygenation reaction.

Although a dioxetane has been suggested as a potential enzyme-bound intermediate in cis-dihydroxylation (Jeffrey et al., 1975), a definitive reaction pathway remains to be elucidated for the aromatic hydrocarbon dioxygenases. Further progress will be contingent on determining the mechanistic branch point between dioxygenase and monooxygenase catalysis and on conducting biophysical studies to define the structural and catalytic properties of the enzyme iron centers.

ACKNOWLEDGMENTS

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REFERENCES

Bernhardt, F. H., Gersonde, K., Twilfer, H., Wende, P., Eckhard, B., Trautwein, A. X., & Pleger, K. (1982) in *Oxygenases and Oxygen Metabolism* (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M. J., Ernster, L., & Estabrook, R. W., Eds.) pp 63–77, Academic, New York.

Billings, R. W., Sullivan, H. R., & McMahon, R. W. (1970) *Biochemistry 9*, 1256–1260.

Boyd, D. R., Sharma, N. D., & Smith, A. E. (1982) *J. Chem. Soc., Perkin Trans. 1*, 2767–2770.

Dagley, S. (1986) in *The Bacteria* (Ornston, L. N., Ed.) Vol. X, pp 527–555, Academic, New York.

Dale, J. A., Dull, D. L., & Mosher, H. H. (1969) *J. Org. Chem. 34*, 2543–2549.

Daly, J. W., Jerina, D. M., & Witkop, B. (1972) *Experientia 28*, 1129–1146.

Davies, J. I., & Evans, W. C. (1964) *Biochem. J. 91*, 251–261.

Englard, S., Blanchard, J. S., & Midelfort, C. F. (1985) *Biochemistry 24*, 1110–1116.

Ensley, B. D., & Gibson, D. T. (1983) *J. Bacteriol. 155*, 505–511.

Ensley, B. D., Gibson, D. T., & Laborde, A. L. (1982) *J. Bacteriol. 149*, 948–954.

Ensley, B. D., Ratzkin, K. L., Osslund, T. D., Simon, M. S., Wackett, L. P., & Gibson, D. T. (1983) *Science (Washington, D.C.) 222*, 167–169.

Fee, J. A., Findling, K. L., Yoshida, T., Hille, R., Tarr, G. E., Hearshen, D. O., Dunham, W. R., Day, E. P., Kent, T. A., & Münck, E. (1984) *J. Biol. Chem. 259*, 124–133.

Finette, B. A., Subramanian, V., & Gibson, D. T. (1984) *J. Bacteriol. 160*, 1003–1009.

Friedrich, E. C., & Taggart, D. B. (1975) *J. Org. Chem. 40*, 720–723.

Gelb, M. H., Heimbrook, D. C., Malkonen, P., & Sligar, S. G. (1982) *Biochemistry 21*, 370–377.

Gibson, D. T., & Subramanian, V. (1984) in *Microbial Degradation of Organic Compounds* (Gibson, D. T., Ed.) pp 181–252, Marcel Dekker, New York.

Gibson, D. T., Koch, J. R., & Kallio, R. E. (1968) *Biochemistry 7*, 2653–2662.

Gibson, D. T., Hensley, M., Yoshioka, H., & Mabry, T. J. (1970) *Biochemistry 9*, 1626–1630.

Gibson, D. T., Gschwendt, B., Yeh, W. K., & Kobal, V. M. (1973) *Biochemistry 12*, 1520–1528.

Gibson, D. T., Yeh, W. K., Liu, T. N., & Subramanian, V. (1982) in *Oxygenases and Oxygen Metabolism* (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M. J., Ernster, L., Estabrook, R. W., Eds.) pp 51–62, Academic, New York.

Groves, J. T., McClusky, G. A., White, R. E., & Coon, M. J. (1978) *Biochem. Biophys. Res. Commun. 81*, 154–160.

Hashimoto, S., Tatsuno, Y., & Kitagawa, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2417–2421.

Hayaishi, O. (1982) in *Oxygenases and Oxygen Metabolism* (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M. J., Ernster, L., & Estabrook, R. W., Eds.) pp 1–12, Academic, New York.

Hayaishi, O., Katagiri, M., & Rothberg, S. (1955) *J. Am. Chem. Soc. 77*, 5450–5451.

Heimbrook, D. C., & Sligar, S. G. (1981) *Biochem. Biophys. Res. Commun. 99*, 530–535.

Horan, J. E., & Schiessler, R. W. (1973) *Org. Synth. 5*, 647–648.

Imuta, M., & Ziffer, H. (1978) *J. Org. Chem. 43*, 4540–4552.

Jeffrey, A., Yeh, H., Jerina, D., Patel, T., Davey, J., & Gibson, D. T. (1975) *Biochemistry 14*, 575–584.

Kobal, V. M., Gibson, D. T., Davis, R. E., & Garza, A. (1973) *J. Am. Chem. Soc. 95*, 4420–4421.

MacDonald, T. L., Burka, L. T., Wright, S. T., & Guengerich, F. P. (1982) *Biochem. Biophys. Res. Commun. 104*, 620–625.

Mason, H. S., Fowlkes, W. L., & Peterson, E. (1955) *J. Am. Chem. Soc. 77*, 2914–2915.

Nordbloom, G. D., White, R. E., & Coon, M. J. (1976) *Arch. Biochem. Biophys. 175*, 524–533.

Serdar, C. M., & Gibson, D. T. (1984) *Abstracts, Annual Meeting of the American Society for Microbiology*, Abstract H148, p 116, American Society for Microbiology, Washington, DC.

Spencer, R. W., Daniels, L., Fulton, G., & Orme-Johnson, W. H. (1980) *Biochemistry 19*, 3678–3683.

Stanier, R. Y., Palleroni, N. J., & Doudoroff, M. (1966) *J. Gen. Microbiol. 43*, 159–271.

Subramanian, V., Liu, T. N., Yeh, W. K., Narro, M., & Gibson, D. T. (1981) *J. Biol. Chem. 256*, 2723–2730.

Subramanian, V., Liu, T. N., Yeh, W. K., Serdar, C. M., Wackett, L. P., & Gibson, D. T. (1985) *J. Biol. Chem. 260*, 2355–2363.

Takikawa, O., Yoshida, R., & Hayaishi, O. (1983) *J. Biol. Chem. 258*, 6808–6815.
Wende, P., Pfleger, K., & Bernhardt, F. H. (1982) *Biochem. Biophys. Res. Commun. 104*, 527–532.
White, R. E., Miller, J. P., Favreau, L. V., & Bhattacharyya, A. (1986) *J. Am. Chem. Soc. 108*, 6024–6031.
Yeh, W. K., Gibson, D. T., & Liu, T. N. (1977) *Biochem. Biophys. Res. Commun. 78*, 401–410.
Ziffer, H., Jerina, D. M., Gibson, D. T., & Kobal, V. M. (1973) *J. Am. Chem. Soc. 95*, 4048–4049.

Deoxycytidylate Hydroxymethylase: Purification, Properties, and the Role of a Thiol Group in Catalysis†

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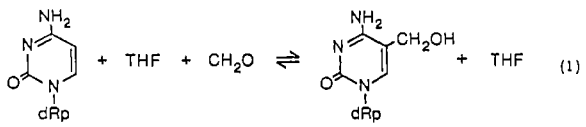
ABSTRACT: Deoxycytidylate (dCMP) hydroxymethylase from *Escherichia coli* infected with a T-4 bacteriophage amber mutant has been purified to homogeneity. It is a dimer with a subunit molecular weight of 28 000. Chemical modification of the homogeneous enzyme with *N*-ethylmaleimide (NEM) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) leads to complete loss of enzyme activity. dCMP can protect the enzyme against NEM inactivation, but the dihydrofolate analogues methotrexate and aminopterin alone do not afford similar protection. Compared to dCMP alone, dCMP plus either methotrexate or aminopterin greatly enhances protection against NEM inactivation. DTNB inactivation is reversed by dithiothreitol. For both reagents, inactivation kinetics obey second-order kinetics. NEM inactivation is pH dependent with a pK_a for a required thiol group of 9.15 ± 0.11. Complete enzyme inactivation by both reagents involves the modification of one thiol group per mole of dimeric enzyme. There are two thiol groups in the totally denatured enzyme modified by either NEM or DTNB. Kinetic analysis of NEM inactivation cannot distinguish between these two groups; however, with DTNB kinetic analysis of 2-nitro-5-thiobenzoate release shows that enzyme inactivation is due to the modification of one fast-reacting thiol followed by the modification of a second group that reacts about 5–6-fold more slowly. In the presence of methotrexate, the stoichiometry of dCMP binding to the dimeric enzyme is 1:1 and depends upon a reduced thiol group. It appears that the two equally sized subunits are arranged asymmetrically, resulting in one thiol-containing active site per mole of dimeric enzyme.

Deoxycytidylate hydroxymethylase (EC 2.1.2.8) catalyzes the reversible hydroxymethylation of dCMP[1] (eq 1). The



(1)

enzyme was discovered in T-even phage infected *Escherichia coli* (Flaks & Cohen, 1957). In 1964, it was semipurified with a low yield (Mathews et al., 1964). THF and CH_2O are required; however, the active cofactor is likely methylenetetrahydrofolate, formed by the rapid condensation of THF and CH_2O (Kallen & Jencks, 1966a,b). The enzyme can be assayed by the measurement of formaldehyde incorporation into product (Flaks & Cohen, 1959) and by ^3H exchange from C-5 of dCMP to solvent (Yeh & Greenberg, 1967). Steady-state kinetic constants have been determined for dCMP, THF, and CH_2O; however, unlike other enzymes (Pogolotti & Santi, 1977) proposed to be mechanistically similar, 5-F-dCMP does not appear to inhibit the crude enzyme (Pizer & Cohen, 1963; Flaks & Cohen, 1959). The objectives of this paper are to show methods for the large-scale production and purification of the enzyme from *E. coli* infected with a T-4 bacteriophage mutant lacking the genes for bacterial cell lysis and to provide chemical modification data implicating the requirement of a single thiol group for catalysis.

Materials and Methods

Materials. Bacteriophage, T-4, 45 am, E-51x5, reg A1x3, and the amber supressor host *E. coli* Cr 63 were generously provided by Dr. John Wiberg of the University of Rochester. Unless specified, all materials were from Sigma Chemical Co. Blue Sepharose CL-6B and Sephadex G-100 and G-25 were from Pharmacia. DEAE-Bio-Gel A and all electrophoresis reagents were from Bio-Rad. Scinti Verse E liquid scintillation cocktail and formaldehyde were from Fisher. *N*-Ethyl[2,3-^{14}C]maleimide was purchased from Amersham. [5-^3H]dCMP and [2-^{14}C]dCMP were obtained from Moravek Biochemicals.

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[1] Abbreviations: dCMP, deoxycytidylate; THF, tetrahydrofolate; MTX, methotrexate (4-amino-10-methylfolic acid); NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; Tris, tris(hydroxymethyl)-aminomethane; dCMP hydroxymethylase, deoxycytidylate hydroxymethylase; DEAE, diethylaminoethyl; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; dUMP, deoxyuridine 5′-phosphate.

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