TOLUENE DIOXYGENASE: A MULTICOMPONENT ENZYME SYSTEM

W. K. Yeh[1], D. T. Gibson and Te-Ning Liu

Department of Microbiology, The University of Texas at Austin 78712

Received August 1,1977

SUMMARY

Pseudomonas putida oxidizes toluene through (+)-*cis*-1(S),2(R)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol). The enzyme catalyzing this reaction was resolved into three protein components. Maximal enzymatic activity was dependent on the presence of ferrous iron and reduced nicotinamide-adenine dinucleotide.

Pseudomonas putida incorporates two atoms of molecular oxygen into benzene to form *cis*-1,2-dihydroxy-1,2-dihydrobenzene (1). The same organism forms *cis*-dihydrodiols from toluene, ethylbenzene, *p*-xylene, *p*-chlorotoluene, *p*-fluorotoluene, *p*-bromotoluene and chlorobenzene (2,3,4,5,6). Analogous *cis*-hydroxylation reactions have been observed in the bacterial metabolism of several aromatic compounds (7).

Studies with isolated enzyme preparations have demonstrated the multicomponent nature of the dioxygenases that form *cis*-dihydrodiols from aromatic substrates. Thus benzene dioxygenase (8) and pyrazon (5-amino-4-chloro-2-phenyl-2H-pridazin-3-one) dioxygenase (9) each contain three components; a flavoprotein, and two non-heme-iron proteins. In this respect they are similar to the alkane monooxygenase system from *Pseudomonas oleovorans* (10). Dioxygenases that hydroxylate the aromatic nucleus and contain at least two components initiate the metabolism of benzoate (11), *o*-phthalate (12) and naphthalene (13).

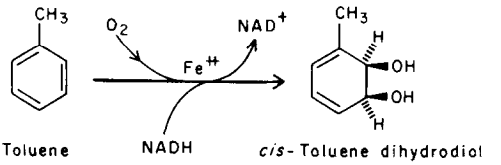
We now describe the resolution of toluene dioxygenase into three

---

[1]Present address, Department of Biology, Yale University, New Haven, Connecticut 06520.

ISSN 0006-291X

protein components. This enzyme system catalyzes the reaction shown below. A preliminary account of this work has been reported (14).



MATERIALS AND METHODS

Materials. (+)-*cis*-1(S),2(R)-Dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) was prepared as described previously (2). Cytochrome c, DEAE-Sephadex-A50, NAD, NADH, NADP and NADPH were obtained from Sigma Chemical Company. DEAE-cellulose was obtained from Whatman Ltd., Springfield Mill, Maidstone, Kent, England. [$^{14}CH_3$]-Toluene (specific activity 26.4 mC/mmole) was from Amersham-Searle Corporation.

Preparation of cell extracts. *Pseudomonas putida* was grown with toluene as the sole source of carbon and energy as described previously for ethylbenzene (3). Cells were harvested by centrifugation and stored at -20 C until required. Frozen cells were suspended in 0.05M KH_2PO_4 buffer, pH 7.2 (1.0 g cells per 10.0 ml buffer) and exposed for 4 min., at 0 C, to the full output of a Bronwill Biosonic III sonic disintegrator. The resulting viscous liquid was treated with deoxyribonuclease for 15 min. prior to centrifugation at 100,000 x g for 1 hr. The clear supernatant liquid was taken as a source of crude cell extract. These preparations showed high endogenous oxygen consumption and could only be assayed by the radioactive procedure described below. Extracts used in polarographic studies were prepared from lyophilized cells as described previously (15). The protein content of cell extracts was determined by the method of Lowry et al. (16).

Enzyme assays. Polarographic measurements were made by the pro-

cedure of Ribbons (17). Each reaction mixture contained in a final volume of 2.0 ml of 0.05M KH_2PO_4 buffer, pH 7.2: $FeSO_4.7H_2O$, 0.2 μmole; NADH, 0.8 μmole; toluene, 0.2 μmole in 0.01 ml of 95% ethanol and cell extract. The enzyme preparation was incubated with $FeSO_4$ for one min. prior to addition to the reaction vessel. The order of addition was, treated enzyme, NADH and toluene.

For enzyme purification studies it was found necessary to develop a more sensitive assay procedure. Thus, toluene dioxygenase activity was determined by measuring the formation of radioactive *cis*-toluene dihydrodiol. Reaction mixtures (0.20 ml) contained in μmoles: NADH, 0.12; $FeSO_4.7H_2O$, 0.15; unlabeled *cis*-toluene dihydrodiol, 1.0; [$^{14}CH_3$]-toluene, 0.014; and cell extract. The reaction was initiated by the addition of [^{14}C]-toluene. After the appropriate time periods (see results) enzymatic reactions were terminated by the addition of 0.01 ml of 1N NaOH, and 0.01 ml of the reaction mixture was applied directly to the base of a silica gel chromatograph sheet (Eastman Chromatogram Sheets, type K130R, silica gel with fluorescent indicator). Chromatograms were developed in chloroform:acetone (80:20) and the reaction product, *cis*-toluene dihydrodiol, was located by viewing under ultraviolet light. The area containing the *cis*-toluene dihydrodiol was cut out and placed in a vial containing 10.0 ml of scintillation fluid (18). Radioactivity in each sample was detected in a Beckman scintillation counter (model LS-100C). Preliminary experiments were conducted to ensure that the reaction was linear with time, that toluene, NADH and Fe^{++} were at saturating levels and that the amount of carrier *cis*-toluene dihydrodiol did not inhibit enzymatic activity.

The enzymatic reduction of cytochrome c was measured at 550 nm as described by Ueda et al. (19). This assay procedure was used to detect cytochrome c reductase activity in cell extracts that was dependent on the presence of partially-purified fraction B (see results). Each re-

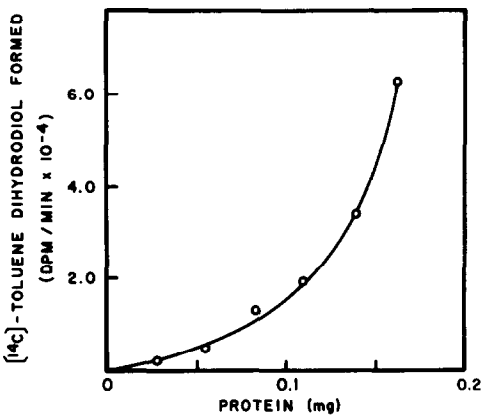


FIGURE 1: Toluene dioxygenase activity as a function of protein concentration. Assays were performed as described in materials and methods.

action mixture (1.0 ml) contained in μmoles: NADH, 0.24; cytochrome c, 0.087; fraction B (70 μg of protein) and 20 μl of each fraction eluted from a DEAE-cellulose column (Fig. 2).

RESULTS

Properties of crude cell extracts. Crude cell extracts oxidized toluene with the consumption of two moles of oxygen per mole of substrate. When enzymatic activity was measured as a function of protein concentration, either polarographically or by measuring product formation (Fig. 1) a linear relationship was not observed. Polarographic studies revealed that enzymatic activity was dependent on the presence of either NAD^+ or NADH. Approximately ten percent of the activity observed with these cofactors was given with NADPH and no activity was observed with $NADP^+$. Maximal enzymatic activity was dependent on the presence of ferrous iron (Table 1).

The measurement of enzymatic activity by oxygen consumption required considerable amounts of protein. Consequently, a more sensitive assay that measures the formation of [^{14}C]- toluene dihydrodiol was devised.

Table 1. Metal ion requirement for toluene dioxygenase activity in crude cell extracts[1].

Metal ion present	Activity nmole O_2/min.
None	62
Fe^{++} ($FeSO_4$)	122
Fe^{+++} ($FeCl_3$)	103
Mg^{++} ($MgSO_4$)	86
Ca^{++} ($CaCl_3$)	66
Cu^{++} ($CuCl_2$)	13
Zn^{++} ($ZnSO_4$)	1

[1]Reaction conditions as described in materials and methods. Each reaction mixture contained dialyzed cell extract (11 mg of protein). All salts were used at a final concentration of 0.1 mM.

Table 2. Requirement of two protein fractions for toluene dioxygenase activity[1].

Fraction added	Activity [^{14}C]-toluene dihydrodiol formed (dpm)
A + B	54,615
A	0
B	0

[1]Reaction mixtures contained in a final volume of 0.20 ml PEG buffer: fraction A, 57 µg of protein; fraction B, 220 µg of protein; $FeSO_4.7H_2O$, 0.15 µmole; NADH, 0.12 µmole; *cis*-toluene dihydrodiol, 1.0 µmole, and [^{14}C]-toluene, 0.014 µmole (813 x 10^3 dpm). Reactions were terminated after 12 minutes and [^{14}C]-toluene dihydrodiol measured as described in materials and methods.

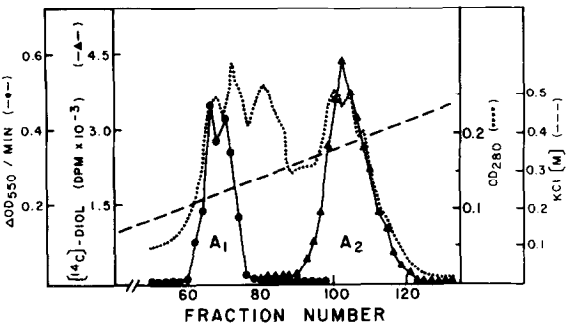


FIGURE 2: Resolution of fraction A by chromatography on DEAE-Sephadex A-50. Fractions (7.5 ml) were collected and assayed for enzymatic activity as described in the text.

This procedure (materials and methods) gave a direct measure of toluene dioxygenase activity and facilitated the localization of individual protein components in column eluates.

Crude cell extracts were unstable and lost 90% of their enzymatic activity over a 48 hour period. No activity was lost over a 72 hour period when 10% ethanol and 10% glycerol were incorporated into 0.05M KH_2PO_4, pH 7.2. This buffer (PEG) was used throughout the purification procedure described below.

Resolution of toluene dioxygenase. Crude cell extract (1200 ml, 8,300 mg of protein) was applied to the top of a column (42 x 4 cm) of DEAE cellulose. Protein was eluted with a linear gradient of 0-0.5M KCl in PEG buffer. Fractions (16.0 ml) were collected and assayed for toluene dioxygenase activity. No enzymatic activity was detected. However, when the contents of tubes 66-93 (fraction A) and 95-105 (fraction B) were combined toluene dioxygenase activity was observed (Table 2). Solutions of fractions A and B were colored yellow-brown and brown respectively.

Partial purification of fraction A. Fraction A (440 ml, 1,340 mg of protein) was diluted three times with PEG buffer and applied to a column (22 x 2 cm) of DEAE Sephadex A-50. The column was washed with two volumes

of PEG buffer prior to the application of one liter linear gradient of 0-0.5M KCl in the same buffer. A yellow protein fraction (A1, tubes 62-74) was detected by its ability to reduce cytochrome c in the presence of fraction B. A brown protein fraction (A2, tubes 94-118) showed toluene dioxygenase activity only in the presence of fractions A1 and B. Fractions A1 and A2, either singly or in combination did not oxidize toluene. The elution profile of fraction A is shown in Figure 2.

Purification of fraction B. Fraction B (175 ml, 122 mg of protein) was diluted three times with PEG buffer and applied to the top of a column (10 x 1.5 cm) of DEAE cellulose. The column was washed with 50 ml of 0.1M KCl in PEG buffer and the brown protein was then eluted with 0.4M KCl in the same buffer. The concentrated solution (15 ml, 62 mg of protein) was dialysed overnight at 4 C against two liters of PEG buffer. Glycerol (2.0 ml) was added to the dialyzed protein solution and the sample subjected to electrophoresis on a 6% polyacrylamide gel (14.0 x 4.0 cm, 200 ml). After 12 hours a brown protein band was observed near the bottom of the gel. This area was removed and the protein recovered electrophoretically (20). This preparation (22 mg of protein), when subjected to analytical polyacrylamide gel electrophoresis (7%) gave a single band that stained for protein. However, we have recently observed that electrophoresis at higher gel concentrations (20%) results in the detection of minor protein components. The further purification and properties of fraction B will be reported elsewhere.

Multicomponent nature of toluene dioxygenase. Partially purified fraction B and the most active samples from fractions A1 and A2 (Fig. 2) were used to demonstrate the multicomponent nature of toluene dioxygenase (Table 3). The resolved system is active in the absence of added ferrous iron although the addition of iron results in a 9-fold increase in activity. Heat treatment (100 C for 5 min.) of fractions A1, A2 and B resulted in a complete loss of enzymatic activity. Fractions A2 and B

Table 3. Multicomponent nature of toluene dioxygenase.

Component omitted	Activity [^{14}C]-toluene dihydrodiol formed (dpm)
None[1]	64,769
Fraction A1	0
Fraction A2	0
Fraction B	0
NADH	0
Fe^{++}	7,323

[1]The complete reaction mixture and assay procedure was as described for Table 2. The amounts of fractions A1, A2 and B were 208, 64 and 35 µg of protein respectively.

were reduced with dithionite and exposed to carbon monoxide. The absorption spectra of these preparations did not reveal the presence of cytochrome P-450.

DISCUSSION

The enzymatic incorporation of molecular oxygen into toluene may occur by oxidation of either the methyl group (21) or the aromatic nucleus (22). Our results indicate that toluene is oxidized at the nucleus to *cis*-toluene dihydrodiol by a multienzyme system that involves at least three components. Similar systems have been described for the monooxygenases that hydroxylate octane and camphor (10,23). Both of these multicomponent complexes contain a flavoprotein that transfers electrons from reduced pyridine nucleotides to a non heme iron protein. A similar situation appears to exist in toluene dioxygenase. The yellow protein (A1) will transfer electrons to cytochrome c. This reaction is significantly stimulated by the presence of the brown protein (B). In re-

sults to be published elsewhere we have demonstrated that protein B contains two moles of iron and two moles of inorganic sulphur per mole of protein. Thus, the component in toluene oxidation appears to be similar to putidaredoxin which functions in the hydroxylation of camphor by *Pseudomonas putida* (24). However, oxygenation in the camphor monooxygenase system is effected by cytochrome P-450_{cam} and none of the resolved toluene components showed the presence of cytochrome P-450. It is conceivable that protein A2 in the toluene system is similar to the ω-hydroxylase of *Pseudomonas oleovorans* (10).

Other multicomponent enzyme systems that catalyze the cis-hydroxylation of aromatic substrates have been reported. All are dioxygenases. Thus, both benzene (8) and pyrazon (9) dioxygenases require a flavoprotein and two non-heme iron proteins for enzymatic activity. Reduced nicotinamide-adenine dinucleotide and ferrous iron are also essential for maximal oxidation of these substrates. Benzoate 1,2-dioxygenase has been resolved into two proteins one of which has NADH-cytochrome c reductase activity (11). The similarity of the toluene dioxygenase enzyme system to these enzymes and the bacterial monooxygenases will become apparent when proteins A1, A2 and B have been purified to homogeneity and their physical properties are determined. Such studies are in progress.

Acknowledgements

This research was supported by Public Health Service Grant ES-00537 from the National Institute of Environmental Health Sciences. Throughout a significant portion of the work DTG was a recipient of a Public Health Service Career Development Award (1 KO4, ES70088) from the National Institute of Environmental Health Sciences and W.K.Y. and T.L. were supported by postdoctoral fellowships from The Robert A. Welch Foundation. We thank Judith M. Davis, J. McKoy and Kit Sang Lam for help with some of the isolation procedures, John E. Rogers for developing the radioactive assay procedure and Roberta DeAngelis for typing the manuscript.

REFERENCES

1. Gibson, D.T., Cardini, G.E. and Kallio, R.E., Biochemistry 9, 1631 (1970).
2. Gibson, D.T., Hensley, M., Yoshioka, H. and Mabry, T.J., Biochemistry 9, 1626 (1970).
3. Gibson, D.T., Yeh, W.K., Gschwendt, B. and Kobal, V.M., Biochemistry 12, 1520 (1973).
4. Davey, J.F., Mahadevan, V. and Gibson, D.T., J. Bacteriol. 119, 930 (1974).
5. Gibson, D.T., Koch, J.R., Schuld, C.L. and Kallio, R.E., Biochemistry 7, 3795 (1968).
6. Ziffer, H., Kabuto, K., Jerina, D.M., Gibson, D.T. and Kobal, V.M., J. Org. Chem. (in press).
7. Gibson, D.T. in Fate and Effects of Petroleum Hydrocarbons in Marine Ecosystems and Organisms, Pergammon Press (in press).
8. Axcell, B.C. and Geary, P.J., Biochem. J. 146, 173 (1975).
9. Sauber, K., Fröhner, C., Rosenberg, G., Eberspächer, J. and Lingens, F. Eur. J. Biochem. 74, 89 (1977).
10. McKenna, E.J. and Coon, M.J., J. Biol. Chem. 245, 3882 (1970).
11. Yamaguchi, M., Yamauchi, T. and Fujisawa, H., Biochem. Biophys. Res. Commun. 67, 264 (1975).
12. Keyser, P. and Ribbons, D.W., Abstr. Annu. Meet. Am. Soc. Microbiol. 126 (1975).
13. Laborde, A.L. and Gibson, D.T., Abstr. Annu. Meet. Am. Soc. Microbiol. 185 (1976).
14. Yeh, W.K. and Gibson, D.T., Abstr. Annu. Meet. Am. Soc. Microbiol. 166 (1974).
15. Gibson, D.T., Koch, J.R. and Kallio, R.E., Biochemistry 1, 2653 (1968).
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., J. Biol. Chem. 193, 265 (1951).
17. Ribbons, D.W., J. Gen. Microbiol. 44, 221 (1966).
18. Kaufman, A.J., Nir, A., Parks, G. and Hours, R.M. in "Tritium in the Physical and Biological Sciences." pp 249-61, I.A.E.A., Vienna (1962).
19. Ueda, T., Lode, E.T. and Coon, M.J., J. Biol. Chem. 247, 2109 (1972).
20. Haschke, R.H. and Campbell, L.L., J. Bacteriol. 105, 249 (1971).
21. Nozaka, J. and Kusunose, M., Agr. Biol. Chem. 33, 962 (1969).
22. Kobal, V.M., Gibson, D.T., Davis, R.E. and Garza, A., J. Am. Chem. Soc. 95, 4420 (1973).
23. Gunsalus, I.C., Hoppe-Seyler's Z. Physiol. Chem. 349, 1610 (1968).
24. Tyson, C.A., Lipscomb, J.D. and Gunsalus, I.C., J. Biol. Chem. 247, 5777 (1972).