Bull. Environ. Contam. Toxicol. (1987) 39:327–333
© 1987 Springer-Verlag New York Inc.

Environmental Contamination and Toxicology

Possible Role of Rat Liver NADPH Cytochrome P-450 Reductase in the Detoxication of DDT to DDD

S. S. A. Zaidi

Department of Biochemistry, National Institute of Communicable Diseases, 22 Sham Nath Marg, Delhi-110 054, India

Pathways for the metabolic disposition of DDT in many rodents have been worked out on the basis of chromatography (Peterson and Robison 1964; Gold and Brunk 1982) and radio-tracer techniques (Datta 1970; Nelson 1970). However, enzymes catalyzing the reactions of metabolic pathway are not well understood. Cytochrome P-450 which is now classified as xenobiotics mono-oxygenase (EC 1.14.19.1) is known to perform a central role in the detoxication of foreign compounds (Coon and Persson 1980), even though its contribution in DDT-detoxication has been poorly known.

Cytochrome P-450 is a multi-enzyme system which has been resolved into three components: cytochrome P-450, NADPH cytochrome c(P-450) reductase and phosphatidylcholine (Lu et al. 1969). The role of cytochrome P-450 in the detoxication of DDT to DDD was suggested by Walker (1969) and later confirmed (Baker and Van dyke 1984; Zaidi and Banerjee 1987). However, it is still unclear whether the numerous activities attributed to cytochrome P-450 system reside in one or more forms of this pigment, or in the other components of this enzyme system. The essential components required for the hydroxylation of a variety of substrates in reconstituted system were reported to be cytochrome P-450, NADPH cytochrome c(P-450) reductase, and phosphatidylcholine (Van der Hoeven 1974; Yasukochi et al. 1979). However, there is a growing awareness that NADPH cytochrome c(P-450) reductase may function in some physiologically important reactions without involving the activity of cytochrome P-450 (Yoshida and Kikuchi 1978; Fukushima et al. 1983).

In continuation of our previous studies, reported recently from this laboratory on the enzymatic detoxication of DDT (Zaidi and Banerjee 1987), this paper provides evidence for the involvement of NADPH cytochrome P-450 reductase in the detoxication of DDT to DDD without involving the activity of cytochrome P-450.

Send reprint requests to S.S.A. Zaidi at National Institute of Occupational Health, Meghani Nagar, Ahmedabad-380016, Gujarat (India).

MATERIALS AND METHODS

DDT and DDD were purchased from Aldrich Chemical Co., Milwaukee WI. These compounds were recrystallized in 95% ethanol to yield a pure substance. Analysis of these compounds by gas chromatography (GC) indicated the purity greater than 99%. Chromosorb WHP 1.5% OV 17 + 1.95% OV 202 was obtained from Packard Instrument BV, Netherlands. DEAE-cellulose D-52 (Whatman, England), calcium hydroxylapatite gel (Sigma Chemical Co., USA) and Sephadex G-200 (Pharmacia Fine Chemicals, Sweden) were used as chromatographic media. Other chemicals used in the study were of analytical reagent grade.

Male albino rats (8 animals) weighing 150-160g were injected with DDT (50mg/kg body weight) every 24 hr for seven days to induce microsomal cytochrome P-450 enzyme system. The animals were maintained on laboratory diet and water ad libitum. Analysis of six random dietary samples by GC showed insignificant level of DDT-residues. Rats were sacrificed by decapitation after 72 hr of the last administered dose. Liver microsomes were prepared and solubilised according to the procedure as described by Comai and Gaylor (1973). All procedures were performed at 4°C except as noted.

The solubilised microsomes (325mg) were immediately chromatographed on a DEAE-cellulose column (20 x 2.2 cm) previously equilibrated with 0.1M tris-buffer, pH 7.6 containing 0.05% deoxycholate, 0.1mM dithiothreitol and 20% glycerol. The column was washed with 200 ml of equilibration buffer and then the protein was eluted with a stepwise gradient of potassium chloride, each prepared in 200 ml of equilibration buffer at a concentration of 0.2M KCl, 0.35M KCl, and 0.6M KCl. Fractions of 5 ml each at a flow rate of 1 ml/min were collected. The eluate was monitored, as described below, for the fractions active in the detoxication of DDT to DDD as well as in the reduction of cytochrome c. The peak reductase fractions eluted with equilibration buffer containing 0.35M KCl were pooled and concentrated by hydroxylapatite column as previously described by Dignam and Strobel (1975). Further purification of the reductase was performed by gel-permeation chromatography using a column of Sephadex G-200. The concentrated protein was applied to Sephadex G-200 column (1.5 x 75 cm) previously equilibrated with 0.1M tris-buffer, pH 7.6 containing 0.05% deoxycholate, 0.1mM dithiothreitol and 20% glycerol. Protein was eluted with equilibration buffer. The flow rate was adjusted to 30 ml/hr and fractions of 3 ml each were collected. Fractions active in the detoxication of DDT to DDD and in the reduction of cytochrome c were localized as described. Active peak fractions were pooled, concentrated by hydroxylapatite column and dialysed against 20 volume of 0.01M tris-buffer, pH 7.8, containing 0.1mM dithiothreitol. The purified reductase was used for subsequent studies.

The enzyme activity for the detoxication of DDT to DDD was measured by GC according to our recently published procedure (Zaidi and Banerjee (1987) and was based on the measurement of DDD as the metabolised product of DDT. In brief, the assay method is as follows. In a final volume of 3.5 ml, 35.2 n mol DDT, dissolved in 25 µl ethanol, was added to an ice cold Erlenmeyer flask (25 ml) that contained 1 ml

enzyme of appropriate concentration, an NADPH generating system (0.59 m mol NADP, 3.75 m mol glucose 6-phosphate and 3.5 Kornberg units of glucose 6-phosphate dehydrogenase), 5.62 m mol $MgCl_2$ and 50 m mol tris-buffer, pH 7.8. Incubation was performed for 1 hr at 37°C under anaerobic condition. The reaction was terminated by 1 ml of $HClO_4$ (20% v/v) and the product DDD and undegraded substrate DDT were extracted three times with 10 vol. of a mixture of hexane and acetone (4:1). The extract was purified by florisil and taken into 2 ml of hexane. An appropriate volume (2 µl) was injected into Packard gas chromatograph. Conditions were: stationary phase, Chromosorb WHP coated with 1.5% OV 17 + 1.95% OV 202; carrier gas, N_2 (120 ml/min); temperature of detector and column, 195°C each; injection port, 220°C; and outlet 200°C. Quantitative analysis of DDD was effected by comparing the peak height with those obtained from a chromatogram of standard DDD with a known concentration. One unit of the enzyme activity was expressed as the amount of the reductase that forms one n mol DDD/hr. Specific activity was defined as unit/mg of protein.

NADPH cytochrome P-450 reductase activity was estimated spectrophotometrically at 550 nm by measuring the reduction of cytochrome c as a convenient and indirect measure of NADPH cytochrome P-450 reductase activity according to the procedure of Master et al (1967). Polyacrylamide gel electrophoresis was carried out in 7.5% acrylamide gel using tris-glycine buffer, pH 8.5 according to the procedure of Davis (1964). Molecular weight determination on native enzyme preparation was carried out using a column of Sephadex G-200.

RESULTS AND DISCUSSION

DDT is partially detoxified to DDD by a process of reductive dechlorination, mediated by the cytochrome P-450 enzyme system. NADPH cytochrome P-450 reductase was separated from the other components of cytochrome P-450 enzyme system and a typical elution profile, obtained after DEAE-cellulose column chromatography, is shown in Fig.1. The reductase was eluted with the equilibration buffer containing 0.35M KCl and was free of cytochrome P-450, cytochrome P-20, cytochrome b_5, and NADH cytochrome b_5 reductase. These impurities were removed into column flow through or by washing the column with 0.2M KCl in equilibration buffer (Dignam and Strobel 1975). Both proteins i.e. cytochrome P-450 (Peak A) and NADPH cytochrome P-450 reductase(Peak B) were found capable of catalysing the detoxication of DDT to DDD (Fig.1). The role of NADPH cytochrome P-450 reductase in the detoxication of DDT to DDD has not been reported earlier. Results of this study also suggest that the reductase is active in the detoxication of DDT to DDD without involving the activity of cytochrome P-450. Similar observations about the functioning of NADPH cytochrome P-450 reductase in some other biochemical reactions without involving the activity of cytochrome P-450 were reported (Yoshida and Kikuchi 1978; Fukushima et al. 1983). The enzyme activity responsible for the detoxication of DDT to DDD, and the activity of NADPH cytochrome P-450 reductase for the reduction of cytochrome c appeared in the same peak (Peak B) and coincided almost exactly. This suggests,

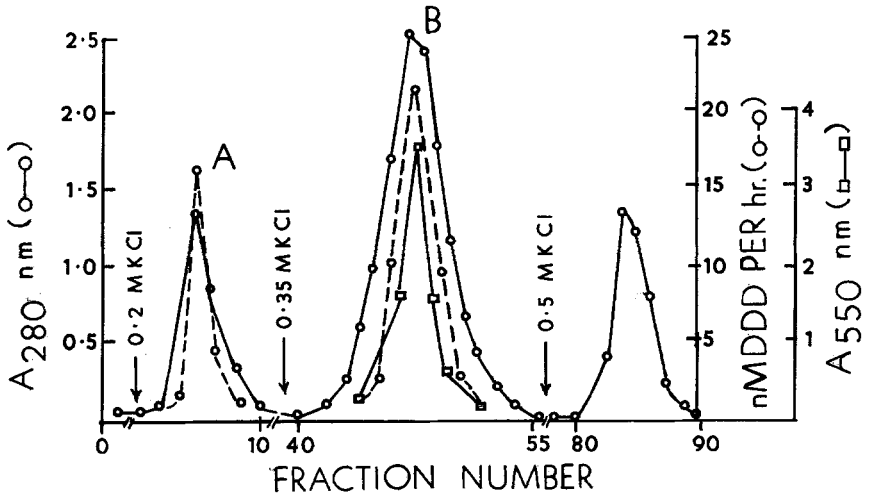

Figure 1. Chromatography of solubilized microsomes on DEAE-cellulose column. Approximately 30 mg of protein was applied to the column and the chromatography was carried out (See Materials and Methods).

the reductase catalyzing the reduction of cytochrome c also catalyzes the detoxication of DDT to DDD. The reductase fractions obtained after DEAE-cellulose chromatography were further purified by gel-permeation chromatography and the results are shown in Fig. 2. Both reductase activities emerged in the same peak.

The purified reductase migrates as a single protein band in polyacrylamide gel electrophoresis. Under the condition of assay, the most purified preparation of the reductase catalyzes the reductive dechlorination of DDT to 17.6 n mol DDD/mg of protein/hr, and the reduction of 42.2 μ mol of cytochrome c/min/mg of protein. The reduction of cytochrome c by the purified NADPH cytochrome P-450 reductase was in close agreement with the values reported by Dignam and Strobel (1975). The molecular weight of the reductase, determined by exclusion chromatography (Fig. 3) was found to be 76,000 daltons which is in accordance with the molecular weight reported for detergent - solubilized preparation of NADPH cytochrome P-450 reductase (Vermilion and Coon 1974; Dignam and Strobel 1975). The optimum pH, 7.8, for the detoxication of DDT to DDD as reported earlier by us (Zaidi and Banerjee 1987) was found to be in close agreement with the pH reported for NADPH cytochrome P-450 reductase which catalyzes the reduction of cytochrome c. Spectral properties of the reductase catalyzing the detoxication of DDT to DDD are in progress. Results of the present study suggest that NADPH cytochrome P-450 reductase detoxifies DDT to DDD.

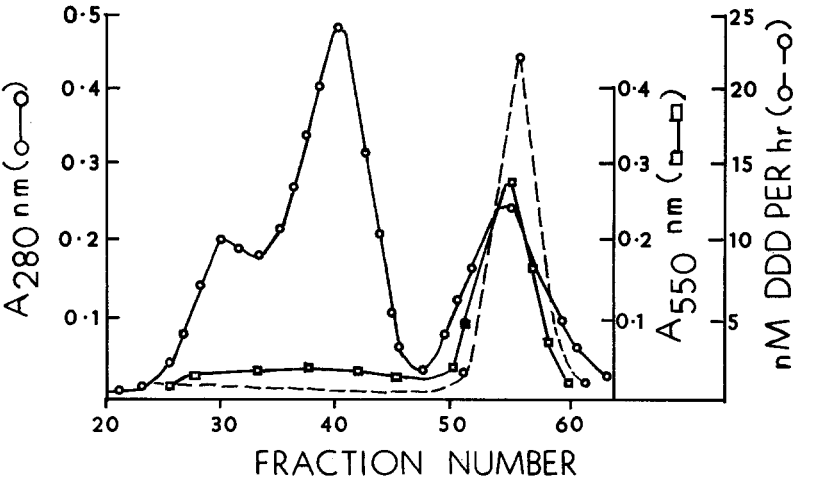

Figure 2. Sephadex G-200 column chromatography of the reductase obtained after DEAE-cellulose column chromatography. Approximately 20 mg of protein was applied to the column and the protein was eluted as described (See Materials and Methods).

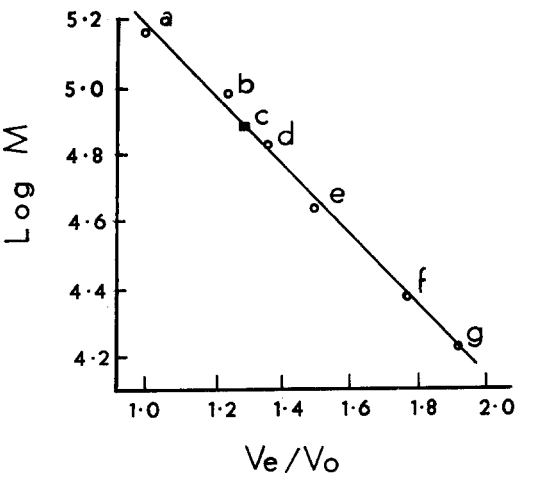

Figure 3. Molecular weight determination of native NADPH cytochrome P-450 reductase by exclusion chromatography. Reference proteins used were: (a) γ-globulin, (b) hexokinase, (d) Albumin, (e) ovalbumin, (f) ribonuclease, and (g) cytochrome c (horse heart). The filled square symbol (c) shows the mean elution volume for NADPH cytochrome P-450 reductase.

Acknowledgments. This work was supported by a grant from the Indian Council of Medical Research, New Delhi. The author is thankful to Dr (Ms) Q Z Hussain for helpful discussions and to the Director, NICD for providing necessary facilities during the course of study.

REFERENCES

Baker MT, Van dyke RA (1984), Metabolism dependent binding of the chlorinated insecticide DDT and its metabolite, DDD, to microsomal protein and lipids. Biochem Pharmacol 33:255-260.

Comai K, Gaylor JL (1973). Existence and separation of three forms of cytochrome P-450 from rat liver microsomes. J Biol Chem 248: 4947-4955.

Coon MJ, Persson AV (1980), Microsomal cytochrome P-450: A central catalyst in detoxication reactions. In: Jacoby WB (ed) Enzymatic Basis of Detoxication, Vol I. Academic Press, New York, p 117.

Datta PR (1970), In vitro detoxication of p,p DDT via p,p DDE to p,p DDA in rats. Toxicology 39:49-58.

Davis BJ (1964). Disc electrophoresis: Methods and application to serum protein. Ann NY Acad Sci 121:404.

Dignam JD, Strobel HW (1975), Preparation of homogeneous NADPH-cytochrome P-450 reductase from rat liver. Biochem Biophys Res Commun 63:845-852.

Fukushima H, Takeda T, Sassaki N, Watanabe T, Nozawa Y (1983), Immunological evidence for the participation of NADPH cytochrome P-450 reductase in microsomal fatty acid acyl Co-A desaturation of Tetrahymena cells lacking in cytochrome P-450. Biochem Biophys Res Commun 115:456-462.

Gold B, Brunk G (1982), Metabolism of 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane and 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane in the mouse. Chem Biol Interact 41:327-339

Lu AYH, Junk KW, Coon MJ (1969), Resolution of the cytochrome P-450 containing-ω-hydroxylation system of liver microsomes into three components. J Biol Chem 244:3714-3721.

Master BSS, Williams CH,Jr, Kamin H (1967), The preparation and properties of microsomal TPNH-cytochrome c reductase from pig liver. In: Estabrook RW and Pullman ME (ed) Methods in Enzymology Vol 10. Academic Press, New York p 565.

Nelson MJ (1970), p,p DDT detoxication by isolated perfused rat liver and kidney. Toxicology 39:195-198.

Peterson JE, Robison WH (1964), Metabolic products of p,p DDT in the rats. Toxicol Appl Pharmacol 6:321-328.

Van der Hoeven TA, Haugen DA, Coon MJ (1974), Preparation and properties of partially purified NADPH cytochrome P-450 reductase from rabbit liver microsomes. Biochem Biophys Res Commun 60:569-574.

Vermilion JL, Coon MJ (1974). Highly purified detergent-solubilized NADPH-cytochrome P-450 reductase from phenobarbital induced rat liver microsomes. Biochem Biophys Res Commun 60:1315-1322.

Walker CH (1969), Reductive dechlorination of p,p-DDT by pigeon liver microsomes. Life Science 8:1111-1115.

Yasukochi Y, Okita RT, Master BSS (1979), Reaction cross-specificities between NADPH cytochrome c(P-450) reductases and cytochrome

P-450 of pig liver and kidney microsomes. In: Yamano TY and Yagi K (ed) Flavins and Flavoproteins, Japan Scientific Press, Tokyo, p 703.

Yoshida T, Kikuchi G (1978), Features of the reaction of heme degradation catalyzed by the reconstituted microsomal heme oxygenase system. J Biol Chem 253:4230-4236.

Zaidi SSA, Banerjee BD (1987), Enzymatic detoxication of DDT to DDD by rat liver: Effects of some inducers and inhibitors of cytochrome P-450 enzyme system. Bull Environ Contam Toxicol 38:449-455.

Received February2, 1987; accepted February 17, 1987