Comparison of Cytochrome P-450- and Peroxidase-Mediated Activations of Carcinogenic Azo Dyes and N-Nitrosamines

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Abstract. Carcinogenic azo dyes (dimethylaminoazobenzene, Sudan I) and N-nitrosamines (N-nitrosomethylaniline, N-nitrosomethylbenzylamine) are oxidized by cytochrome P-450 isoenzymes and peroxidase yielding metabolites which *in vitro* bind to DNA and transfer RNA (tRNA). The parallelism and differences in oxidative reactions are described. Peroxidase is more effective than P-450 in activating reactions of some carcinogens studied. The presence of either of these enzymes is supposed to be responsible for the organ and/or cell type specific effects of the carcinogens studied.

Key words: Activation of carcinogens — Azo dyes — N-nitrosamines — Cytochrome P-450 — Peroxidase

Abbreviations: AB, 4-aminoazobenzene, DAB, N,N-dimethyl-4-aminoazobenzene; HPLC, high performance liquid chromatography; MFO, mixed-function-oxidase; MAB, N-methyl-4-aminoazobenzene; NMA, N-nitrosomethylaniline; NMBA, N-nitrosomethylbenzylamine; TLC, thin layer chromatography; tRNA, transfer RNA; R_F, relative mobility.

Introduction

The mixed-function-oxidase (MFO) with cytochrome P-450 as a terminal oxidase is primarily directed against xenobiotics (detoxication). However, the MFO system is also supposed to be responsible for a process known as bioactivation of xenobiotics. The explanation for both processes (detoxication and activation) catalyzed by MFO system probably is that activation is due to one (or more) specific isoenzyme

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of cytochrome P-450, while other isoenzymes catalyze detoxication reactions In particular, the oxidation of carcinogenic compounds has been a focus of scientific studies The amounts of reactive intermediates formed are supposed to be the result of two mutually competing reactions (detoxication and activation) catalyzed by different types of hemoproteins, so that not only the organ and/or cellular levels but also the cytochrome P-450 isoenzyme population will determine whether a compound will be activated or detoxicated (Ioannides and Parke 1990)

In addition, it not only are the isoenzymes of cytochrome P-450 which are supposed to be responsible for both processes, other enzymes are also known to be implicated in the activation or detoxication reactions of several xenobiotics (Guengerich 1990) This has mainly been assumed for extrahepatic tissues, which are not rich in cytochromes P-450

The focus of the present study was the activation of selected carcinogens catalyzed by specific isoenzymes of cytochrome P-450 as well as by another hemoprotein, peroxidase Two classes of carcinogens, activated by different isoenzymes of cytochrome P-450, which selectively induce malignant tumors in different organs, were studied Sudan I (1-phenylazo-2-hydroxynaphthalene), a model non-aminoazo dye, N,N-dimethylaminoazobenzene (DAB), an aminoazo dye, N-nitrosomethylaniline (NMA) and N-nitrosomethylbenzylamine (NMBA), N-nitroso compounds, were chosen as carcinogens inducing tumors in different tissues and examples of compounds activated by different cytochrome P-450 isoenzymes (Garner et al 1984, Stiborová et al 1988*a*, Guengerich 1990, Ioannides and Parke 1990, Yang et al 1990) The parallelism as well as differences in activation processes catalyzed by specific cytochrome P-450 isoenzymes and peroxidase are presented

Materials and Methods

Chemicals and radiochemicals

Chemicals were obtained from the following sources β-naphthoflavone from Aldrich Chemical Co , Milwaukee, WI, USA, NADH, NADPH, DNA (from calf thymus), peroxidase (horseradish) from Boehringer, Mannheim, FRG, Sudan I from british Drug Houses, Poole, UK, dimethylaminoazobenzene, methylaminoazobenzene and aminoazobenzene from Merck, Darmstadt, FRG, N-nitrosomethylaniline and N-nitrosomethylbenzylamine were synthesized as described by Druckrey et al (1967), rat liver tRNA was prepared according to Rogg et al (1969), all other chemicals were reagent grade or higher

^{14}C-labeled Sudan I (0 54 mCi/mmol) was synthesized from [U-^{14}C] aniline (2 5 mCi/mmol, Ammersham International plc, Amersham, UK) and non labeled β-naphthol (Matrka and Pípalová 1982) ^{14}C-labeled DAB (0 5 mCi/mmol) was prepared from radioactive aniline and non labeled N,N-dimethylaniline (Matrka and Pípalová 1982) Radioactive aniline was also used to prepare ^{14}C-labeled N-nitrosomethylaniline by the procedure described by Grandjean et al (1976) All the ^{14}C-labeled compounds were purified by column chromatography on basic alumina and on a thin layer of Silicagel G (Woelm) The labeled compounds were stored at -17 °C

Subcellular preparations

Male Sprague-Dawley rats (about 100–150 g) were injected i p with β-naphthoflavone dissolved in maize oil (60 mg/kg body weight) once a day for 3 consecutive days The animals were starved for 16–18 h prior to being killed, and liver microsomes containing cytochrome P-450 were prepared as described by Kimura et al (1982) and stored at −70 °C

Incubations

Incubation mixtures used for the modification of DNA by radiolabeled azo dyes activated by microsomal cytochrome P-450 contained in a final volume of 1 5 ml 50 mmol/l potassium phosphate buffer pH 7 7, 2 mmol/l NADPH, 3 5 mg microsomal proteins, 0 2 mmol/l ^{14}C-Sudan I or ^{14}C-DAB dissolved in methanol (100 μmol/l 5 ml incubation), 1 mg DNA

Incubation mixtures used for the modification of DNA or tRNA by radiolabeled azo dyes or ^{14}C-nitrosomethylaniline activated by peroxidase contained in a final volume of 1 5 ml 50 mmol/l Tris-HCl (pH 7 46–8 4), 0 2 mg horseradish peroxidase, 1 0 mmol/l H_2O_2, 0 2 mmol/l radiolabeled azo dyes or 0 825 mmol/l ^{14}C-N-nitrosomethylaniline (dissolved in dimethylsulfoxide, 50 μl/l 5 ml incubation), and 1 mg DNA or tRNA After incubation (37 °C, 60 min), the mixtures were extracted twice with ethyl acetate (2 × 2 ml) After evaporation of the ethyl acetate extracts to yield dry residue, the latter was dissolved in a minimal volume of methanol and chromatographed on a thin layer of silica gel (Schleicher and Schuell, FRG) Details of the thin layer chromatography of ^{14}C-Sudan I and its metabolites formed by microsomal cytochrome P-450 or peroxidase have been described previously (Stiborová et al 1988 a, b, c, 1991) The chromatograms with ^{14}C-DAB separated from its metabolites were developed using a mixture of diethyl ether/*n*-hexane (3 1, v/v), the products were mechanically separated from the layers, placed into scintillation vials, and the radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2000 CA) Standards (methylaminoazobenzene, aminoazobenzene) were processed identically The oxidation products of N-nitrosomethylaniline of N-nitrosomethylbenzylamine were determined by a different procedure (see under Oxidation of N-nitrosamines by the peroxidase system)

DNAs or tRNAs modified by metabolites of radioactive carcinogens formed by both activating enzymatic systems were isolated by the phenol/chloroform procedure as described earlier (Stiborová et al 1988 b, c, 1990 a, b) The ^{14}C radioactivity of DNA or tRNA was measured by liquid scintillation counting (Packard Tri-Carb 2000 CA) The content of nucleic acids was measured spectrophotometrically (Rogg et al 1969, Yamazoe et al 1988)

Oxidation of N-nitrosamines by the peroxidase system

The reaction mixture contained in a final volume of 3 0 ml 50 mmol/l potassium phosphate buffer pH 7 7, 200 μg horseradish peroxidase, 1 mmol/l H_2O_2 and 0–3 mmol/l N-nitrosomethylaniline or N-nitrosomethylbenzylamine After 30 min incubation (37 °C), 2 ml aliquots of the mixtures were reacted with 2,4-dinitrophenylhydrazine in orthophosphoric acid The 2,4-dinitrophenylhydrazones formed were extracted with *n*-hexane CH_2Cl_2 (9 1, v/v), and the organic solvents were evaporated under a stream of N_2 to dryness The contents of 2,4-dinitrophenylhydrazones formed from aldehydes raised from N-nitrosamines were determined by HPLC as described by Janzowski et al (1982)

Measurement of NADH oxidation and oxygen consumption

The reaction mixture contained in a final volume of 2 ml: 50 mmol/l potassium phosphate buffer pH 7.46–8.4; 0.1 mmol/l NADH; 0–0.0114 mmol/l H_2O_2; 0–5 μg horseradish peroxidase; and 0–1.2 mmol/l carcinogen. Reactions were started by addition of the respective carcinogen, and gradual disappearance of NADH was followed at 340 nm using a Beckmann D-62 spectrophotometer. Oxygen consumption was measured with a Clark-type electrode.

Results and Discussion

The carcinogenic effects of the agents studied are organ specific. The target organs include liver (DAB, Sudan I), urinary bladder (Sudan I) esophagus (N-nitrosomethylaniline, N-nitrosomethylbenzylamine), and lungs (N-nitrosomethylaniline, N-nitrosomethylbenzylamine) (Garner et al. 1984; Dipple et al. 1987; Preussmann and Wiessler 1987; Stiborová et al. 1988 a; b; Guengerich 1990). Hepatic, esophageal and some pulmonary cells are rich in cytochromes P-450 which, moreover, show induction patterns in these organs. The urinary bladder and other pulmonary cells, on the other hand are not abundant in these enzymes; they however contain large quantities of another hemoprotein, peroxidase (Preussman and Wiessler 1987; O'Brien 1988; Guengerich 1990; Ioannides and Parke 1990; Schuller et al. 1990; Yang et al. 1990). Cytochrome P-450 and peroxidase were therefore studied from the point of view of their ability to oxidize the carcinogens yielding active intermediates that bind to DNA.

Oxidation of azo compounds (Sudan I, DAB) and N-nitrosamines (NMA, NMBA) by cytochrome P-450

The oxidation of azo compounds by cytochrome P-450 was extensively studied previously (for a review see Ioannides and Parke 1990; Levine 1991). DAB and Sudan I are preferentially oxidized by cytochrome P-450 induced by β-naphthoflavone or polycyclic aromatic hydrocarbons (cytochromes P-448 or cytochrome P-450c, P-450d) (Ioannides and Parke 1990; Stiborová et al. 1988 a).

From the point of view of chemical toxicity, one of the most important differences between cytochrome P-448 and other cytochrome P-450 isoenzymes are their contrasting roles in metabolic activation and detoxication of carcinogens. Cytochromes P-448 almost always activate the substrates to yield reactive metabolites resistant to subsequent conjugation (e.g. with glucuronic acid, sulphate, glutathions etc.), which interact with DNA givig rise to carcinogenicity (Ioannides and Parke 1990).

Both DAB and Sudan I are detoxicated by cytochrome P-448 and activated to electrophiles (Kimura et al. 1982; Kadlubar 1987; Stiborová et al. 1988 a; b; Ioannides and Parke 1990).

N-demethylation and N-hydroxylation of DAB catalyzed by cytochromes P-448 are reactions resulting in the activation of this carcinogen. Cytochrome P-450d catalyzes N-hydroxylation at twice the rate of cytochrome P-450c (Kimura et al. 1982). The ultimate carcinogen are nitrenium or carbenium ions formed by splitting of sulphate conjugates (Kadlubar 1987).

Activation of Sudan I by cytochrome P-448 was studied in one of our previous works. Oxidative splitting of the azo group, leading to the formation of benzenediazonium ion, is supposed to be the activating reaction (Stiborová et al. 1988 a; b).

The characteristics of binding of azo dyes metabolites, formed by cytochrome P-448, to DNA are shown in Table 1.

Table 1. Oxidation of ^{14}C-DAB and ^{14}C-Sudan I by rat liver microsomal cytochrome P-448 and binding to DNA

Activating system	DNA binding nmol/mg of DNA		Converted compounds (%)	
	DAB	Sudan I	DAB	Sudan I
Complete microsomal cyt. P-448 system, pH 7.7 (P-448, NADPH, compound)	0.41 ± 0.01 (1.3 adducts in 10^4 nucleotides)	0.38 ± 0.02 1.2 adducts	90.0 ± 2.9	83.8 ± 3.5
In absence of cyt. P-448	0	0	0	0
In absence of NADPH	0.01 ± 0.001	0.1 ± 0.005	3.8 ± 0.3	5.1 ± 0.5

For experimental conditions see Materials and Methods. The values shown are averages and standard deviations from triplicate determinations.

The activation of carcinogenic nitrosamines by cytochrome P-450 is mainly associated with alcohol-inducible cytochrome P-450j and partially with cytochromes P-450 induced by phenobarbital (Ioannides and Parke 1990; Yang et al. 1990). α-C-Hydroxylation of N-nitrosomethylbenzylamine is responsible for the formation of methyl or benzyl-diazonium ions, while α-C-hydroxylation of N-nitrosomethylaniline results in the release of benzenediazonium ions. These can cause covalent modification of DNA (Dipple et al. 1987; Preussmann and Wiessler 1987). Other specific activating reactions are, however, not excluded (Dipple et al. 1987; Preussmann and Wiessler 1987; Schuller et al. 1990).

Oxidation of azo compounds (Sudan I, DAB) and N-nitrosamines (NMA, NMBA) by peroxidase

^{14}C-DAB is oxidized by peroxidase in the presence of H_2O_2 yielding six different products Two of the products showed identical chromatographic mobility with N-demethylated derivatives of DAB (N-methylaminoazobenzene, aminoazobenzene) The identity of further four products has not yet been established (Table 2)

Table 2. Products formed ^{14}C-DAB by the peroxidase/H_2O_2 system

TLC in diethyl ether/ *n*-hexane (3 1) R_f	Products[a] obtained by incubations			Corresponding standard
	in absence of peroxidase and presence of H_2O_2	in absence of both peroxidase and H_2O_2	in presence of both peroxidase and H_2O_2	
0 01	1 3 ∓ 0 1	0 2 ∓ 0 01	55 01 ∓ 0 70	unknown
0 16	–	–	3 98 ∓ 0 31	unknown
0 28	–	–	4 27 ∓ 0 40	unknown
0 40	–	–	2 90 ∓ 0 30	unknown
0 70	–	–	4 97 ∓ 0 50	aminoazobenzene (AB)
0 75	0 2 ∓ 0 02		14 09 ∓ 0 70	methylaminoazo-benzene (MAB)
0 80	98 5 ∓ 0 96	99 8 ∓ 0 98	14 78 ∓ 0 6	DAB

[a] ^{14}C-labeled products and ^{14}C-DAB were extracted from incubation mixtures by ethylacetate and separated by TLC (see Methods) The values shown are percentages of total radioactivity Averages and standard deviations from triplicate determination

– not formed

Ring hydroxylated products of Sudan I were found to be formed by peroxidase as minor products, the benzenediazonium ion and other so far unknown products as the major ones (Stiborová et al 1988 c, 1991)

Both N-nitrosamines are oxidized by peroxidase, NMA being a better substrate to the enzyme than NMBA Products of α-C-hydroxylating reactions were the same as detected in reactions catalyzed by cytochrome P-450 (Kroeger-Koepke et al 1981) (Table 3) Moreover, the amounts of aldehydes (formaldehyde, benzaldehyde) formed by the peroxidase system were in the same range as those formed by cytochrome P-450 (Tu and Yang 1983, Yang et al 1990) It is not only the formation of alkyl- or aryl-diazonium ions during NMA and NMBA oxidation which is responsible for initiation of carcinogenic processes The production of formaldehyde may also have significant physiological effect because of its carcinogenicity

Table 3. Oxidation of N-nitrosomethylaniline and N-nitrosomethylbenzylamine by peroxidase

Concentrations of nitrosamines in reaction mixture (mmol/l)	Rate of oxidation (nmoles aldehyde formed/min/mg peroxidase)		
	NMA	NMBA	
	formaldehyde	formaldehyde	benzaldehyde
0	0	0	0
0 5	0 72 ∓ 0 02	0 015 ∓ 0 002	0 19 ∓ 0 02
1 0	1 10 ∓ 0 09	0 13 ∓ 0 01	0 29 ∓ 0 02
1 5	1 20 ∓ 0 09	0 40 ∓ 0 03	0 59 ∓ 0 04
2 0	1 3 ∓ 0 10	0 65 ∓ 0 05	1 14 ∓ 0 09
3 0	1 44 ∓ 0 10	0 89 ∓ 0 06	1 27 ∓ 0 10

For experimental conditions see the text Values shown represent means and standard deviations from three separate experiments

Table 4. Oxidation of NADH and oxygen uptake catalyzed by the peroxidase/H_2O_2 carcinogen system

Carcinogen (mmol/l)	Converted NADH (nmol NADH/min/μg peroxidase)	Oxygen uptake (nmolO_2/min/μg peroxidase)
none	0 04 ∓ 0 006	0 008 ∓ 0 001
Sudan I (0 3 mmol/l)	5 65 ∓ 0 200	0 008 ∓ 0 001
DAB (0 3 mmol/l)	6 25 ∓ 0 300	5 000 ∓ 0 080
NMA (1 2 mmol/l)	1 78 ∓ 0 050	1 420 ∓ 0 040
NMBA (1 2 mmol/l)	0 23 ∓ 0 002	0 022 ∓ 0 003

For experimental conditions see the text The dissappearance of NADH was recorded spectrophotometrically at 340 nm Oxygen consumption was measured with a Clark type electrode Values shown are averages and standard deviations of three parallel experiments

(Guengerich 1990)

Although some of the products formed from carcinogens under the activity of peroxidases are identical with those formed by cytochromes P-450, the mechanisms of reactions catalyzed by the two enzyme systems are different N-demethylation catalyzed peroxidases results in the formation of free radical cation and iminium cation by sequential one-electron oxidations, the iminium cation is then hydrolyzed to formaldehyde and demethylated amine (Guengerich 1990) This mechanism however, has not been confirmed for N-demethylation with all carcinogens studied

The carcinogens studied were found to be oxidized by peroxidase to reactive

metabolites with NADH oxidation activity (Table 4). In addition, DAB and NMA mediated an effective oxygen uptake under the conditions used (Table 4). These findings (the ability of metabolites to oxidize NADH) could imply that the reactions are one-electron redox processes having free radicals as the primary products, similarly as observed with several other xenobiotics oxidized by peroxidase to radicals (O'Brien 1988; Stiborová and Anzenbacher 1990).

Table 5. Dependence of the carcinogens binding to DNA and tRNA on time of addition of nucleic acids into reaction mixtures.

Time of addition of nucleic acids into the reaction medium (min)		DNA binding (nmol/mg)	DNA binding (adducts in nucleotides)	tRNA binding (nmol/mg)	tRNA binding (adducts in nucleodites)
DAB	0	2.30 ∓ 0.20	7.5 in 10^4	14.06 ∓ 0.07	4.6 in 10^3
	10	0.10 ∓ 0.02	3.3 in 10^5	1.85 ∓ 0.02	6.0 in 10^4
Sudan I	0	4.10 ∓ 0.30	1.3 in 10^3	14.00 ∓ 0.30	4.6 in 10^3
	15	0.05 ∓ 0.008	1.6 in 10^5	1.41 ∓ 0.30	4.6 in 10^4
NMA	0	0.60 ∓ 0.10	2.0 in 10^4	3.01 ∓ 0.12	1.0 in 10^3
	15	–	–	0.30 ∓ 0.02	1.0 in 10^4

For experimental conditions see the text. The values shown are averages and standard deviations of three experiments.

This suggestion is also supported by the finding of very reactive metabolites with short half-lives binding to DNA or tRNA, during reactions catalyzed by peroxidase, the binding being more intensive than with cytochromes P-450 (Tables 1 and 5). No similar findings (NADH oxidation, rapid modifications of nucleic acids) could be noted in reactions with carcinogens being activated by cytochrome P-450 (results not shown). The absence of rapid DNA modification as well as the inability of carcinogen products to oxidize NADH in the cytochrome P-450 system could also confirm the view that peroxidase (the radical mechanism) and cytochrome P-450 (the non-radical mechanism) catalyze different processes.

The present results show that isoenzymes of cytochrome P-450 are not the only to play the key role in activation of toxic (carcinogenic) chemicals. The hemoprotein peroxidase is also very effective in this activation. Moreover, both activating enzymes may interact in the organism and this will determine whether the chemical agents become activated or detoxicated.

The actual mechanisms responsible for the initiation of chemical carcinogenesis in different organisms and/or cells are difficult to define. In tissues and/or cells rich in cytochrome P-450 (e.g. liver), carcinogens may be activated by these enzymes and then transported (as active metabolites or their conjugates) into target

organs and/or cells. Moreover, conjugate-forming enzymatic reactions may have various capacities in different organs (Preussmann and Wiessler 1987). On the other hand, in tissues (or cells), which are not rich in cytochromes P-450, other specific activating systems are suggested to be involved in the activation of carcinogens (Preussmann and Wiessler 1987; O'Brien 1988; Guengerich 1990; Schuller et al. 1990). Peroxidases have been supposed to be among these activating enzymes (O'Brien 1988; Yamazoe et al. 1988; Guengerich 1990; Schuller et al. 1990).

Our present results may serve to support the hypothesis that organ or cell specificity of different carcinogens may also be due to the enzymatic activity of peroxidases. *In vivo* studies are needed to extend the knowledge on the participation of peroxidases in processes underlying differences in organ specificity of carcinogen.

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