Biochemical Pharmacology, Vol. 40, No. 11, pp. 2415–2423, 1990.
Printed in Great Britain.

0006-2952/90 $3·00 + 0.00

CHARACTERISTICS OF TWO CLASSES OF AZO DYE REDUCTASE ACTIVITY ASSOCIATED WITH RAT LIVER MICROSOMAL CYTOCHROME P450

SHMUEL ZBAIDA and WALTER G. LEVINE*
Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A.

(*Received* 24 *November* 1989; *accepted* 1 *June* 1990)

Abstract—Azo dyes are reduced to primary amines by the microsomal enzymes NADPH–cytochrome P450 reductase and cytochrome P450. Amaranth, a highly polar dye, is reduced almost exclusively by rat liver microsomal cytochrome P450 and the reaction is inhibited almost totally by oxygen or CO. Activity is induced by pretreatment with phenobarbital or 3-methylcholanthrene. In contrast, microsomal reduction of the hepatocarcinogen dimethylaminoazobenzene (DAB), a lipid soluble, weakly polar compound, is insensitive to both oxygen and CO. However, reconstitution of activity with purified NADPH–cytochrome P450 reductase and a partially purified cytochrome P450 preparation indicates that activity is catalyzed almost exclusively by cytochrome P450. Activity is induced by clofibrate but not phenobarbital, β-naphthoflavone, 3-methylcholanthrene, isosafrol, or pregnenolone-16α-carbonitrile. These observations suggest the existence of at least two classes of azoreductase activity catalyzed by cytochrome P450. To investigate this possibility, the reduction of a number of azo dyes was investigated using microsomal and partially purified systems and the characteristics of the reactions were observed. Microsomal reduction of azo dyes structurally related to DAB required a polar electron-donating substituent on one ring. Activity was insensitive to oxygen and CO if the substrates had no additional substituents on either ring or contained only electron-donating substituents. Introduction of an electron-withdrawing group into the prime ring conferred oxygen and CO sensitivity on the reaction. Substrates in the former group are referred to as insensitive and substrates in the latter group as sensitive. Inhibitors of cytochrome P450 activity depressed reduction of both insensitive and sensitive substrates. In a fully reconstituted system containing lipid, highly purified NADPH–cytochrome P450 reductase and a partially purified cytochrome P450 preparation, rates of reduction of various insensitive substrates varied several-fold, whereas rates of reduction of sensitive substrates varied by three orders of magnitude. Using purified enzymes, each of the insensitive substrates was shown to be reduced by reductase alone, but only at a fraction of the rate seen in the fully reconstituted system, implying that reducing electrons were transferred to the dyes mainly from cytochrome P450. Conversely, there was substantial, in some cases almost exclusive, reduction of sensitive substrates by purified reductase alone and almost no inhibition by CO. Their reduction, however, was inhibited by CO in microsomal systems. This apparent discrepancy may be attributed to less effective coupling between reductase and cytochrome P450 in the reconstituted system compared to that in microsomes, permitting "leakage" of electrons from flavoprotein directly to substrate. Microsomal reduction of sensitive substrates was induced by treatment with phenobarbital, isosafrole, and pregnenolone-16α-carbonitrile or clofibrate, whereas reduction of insensitive substrates was induced only by clofibrate, suggesting catalysis by a more restrictive group of cytochromes P450. It is concluded that both sensitive and insensitive substrates are reduced by cytochrome P450. Observations on differential induction and sensitivity to oxygen and CO imply that at least two distinct species of cytochrome P450 catalyze the reduction of insensitive and sensitive substrates. Speculation is also made of the significance of the differences of oxygen and CO sensitivity.

N-Demethylation, N-oxidation and esterification are implicated in the activation of certain azo dyes to primary carcinogenic agents, whereas protective action is generally associated with reductive cleavage of azo bonds to primary amines [1]. Reduction of 4-dimethylaminoazobenzene (DAB) is catalyzed by a form of microsomal cytochrome P450 which is selectively induced by clofibrate *in vivo* as well as in primary hepatocyte culture [2–5], but which is distinct from the clofibrate-induced form which catalyzes laurate hydroxylation [6, 7]. The reaction is insensitive to oxygen and CO [5]. However, evidence obtained with selective inhibitors and reconstitution of a partially purified mixed-function oxidase system strongly supports cytochrome P450 as the catalytic agent [4, 5]. Structure–activity studies with related aromatic azo dyes indicate that a polar, electron-donating substituent at the 4 position is essential for binding to cytochrome P450 and enzymic reduction [8]. Thus, compounds such as azobenzene and *p*-isopropylazobenzene do not exhibit typical substrate binding spectra in the presence of microsomes and are not reduced. The requirement for electron-donating property is illustrated by the fact that aminoazobenzene is readily reduced by liver microsomes, whereas its benzoyl derivative is not. The strong electron-attracting ability of the carbonyl group in the latter compound counteracts

* Correspondence: Dr. Walter G. Levine, Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

the electron-donating property of the parent primary amine.

The reduction of some azo dyes is oxygen sensitive. Mason and his colleagues [9, 10] found that sulfonazo III and arsenazo III are reduced anaerobically by hepatic microsomes. One-electron reduced intermediates are formed which immediately reoxidize under aerobic conditions to the parent azo compound with the formation of superoxide anion free radical. Neoprontosil and neotetrazolium are reduced anaerobically by microsomal NADPH–cytochrome P450 reductase and by cytochrome P450 [11]. The portion attributed to cytochrome P450 is inhibited by CO. Reduction of amaranth by rat liver microsomes is suppressed by antibodies to cytochrome P450 and is inhibited virtually 100% by CO [12, 13]. The activity is attributed entirely to cytochrome P450. Its reduction has been reported to be oxygen insensitive by one group [14], but sensitive by others [12, 15].

This laboratory has reported that DAB is also reduced by purified NADPH–cytochrome P450 reductase, but the activity is only a fraction of that in the fully reconstituted cytochrome P450 system [5]. Reduction by flavoprotein alone is inhibited completely by oxygen although reduction by the reconstituted system is not. The cytosolic enzyme, NAD(P)H:quinone reductase (DT-diaphorase), also catalyzes the reduction of certain azo dyes [16]. It contains two FAD per molecule and can use either NADPH or NADH as sources of reducing electrons. Reduction is oxygen insensitive, which is attributed to a two-electron reduction step, bypassing the oxygen-sensitive free radical formed during a one-electron reduction. The cytosolic enzyme reduces methyl red (2′-carboxy-DAB) but not DAB itself [16]. The present study describes two classes of cytochrome P450-catalyzed azoreduction based on structural requirements, CO and oxygen sensitivity and response to inducing agents.

EXPERIMENTAL PROCEDURES

Instrumentation

Nuclear magnetic resonance (NMR) studies were performed on a Varian VXR 60 MHz spectrometer. All samples were dissolved in deuterated chloroform using tetramethylsilane as an internal standard. All chemical shifts are given downfield from the standard. Mass spectral analyses were performed on a VG-7070E mass spectrometer at 70 eV. Fluorometric measurements were performed on a Perkin–Elmer Fluorescence Spectrophotometer, model MPF-3L. Various ratios of oxygen versus nitrogen were obtained using a Tylan mass flow controller.

Chemicals

4-Hydroxyazobenzene, *o*-methyl red [2-(4-dimethylaminophenylazo)benzoic acid], 4-(4-dimethylaminophenylazo)benzene arsonic acid hydrochloride, methyl orange, 4-(2-pyridylazo)*N,N*-dimethylaniline, methyl anthranilate, metyrapone, and α-naphthoflavone were purchased from Aldrich (Milwaukee, WI). Piperonylbutoxide was obtained from Fluka (Ronkonkoma, NY). *p*-Methyl red [4-(4-dimethylaminophenylazo)benzoic acid sodium salt, octylamine and 4-aminoazobenzene were bought from Eastman (Rochester, NY). DAB (4-dimethylaminoazobenzene) was purchased from Sigma (St. Louis, MO). 4-Dimethylamino-4′-hydroxyazobenzene was synthesized as previously described [17]. 4-Methylaminoazobenzene was a gift of Dr. Fred Kadlubar, National Center for Toxicological Research (Jefferson, AR). SKF 525-A was a gift of Smith Kline & French Laboratories (Philadelphia, PA).

Esterification of ortho- *and* para-*methyl red*

Esters were prepared according to previously described procedures [18] by refluxing the azo compounds (0.01 mol) for 5 hr with an excess (enough to dissolve the azo dye) of analytical methyl alcohol in the presence of a small amount of concentrated sulfuric acid as a catalyst. The excess methanol was removed by distillation. The residue was dissolved in 60 mL water, made alkaline with sodium hydroxide, and extracted (3×50 mL) with chloroform. The combined extracts were washed several times with water until neutral to litmus paper, dried over anhydrous sodium sulfate, and evaporated.

o-*Methyl red methyl ester.* 2-(4-Dimethylaminophenylazo)methyl benzoate was recrystallized from methanol water, 56% yield, m.p. = 107–108°. A melting point of 94.5–96° was reported after recrystallization from hexane–carbon tetrachloride–chloroform [19]. NMR: δ8.00–6.65 8H m, 3.85 3H s, 3.05 6H s. Molecular weight (calcd.) 283; found (MS): $m/z = 283$ (25%, M^+), 252 (5%, M^+-OCH_3), 148 (10%, M^+-$C_6H_4CO_2CH_3$), 135 (10%, $C_6H_4CO_2CH_3$), 120 [100%, $C_6H_4N(CH_3)_2$].

p-*Methyl red methyl ester.* 4-(4-Dimethylaminophenylazo)methyl benzoate was recrystallized from methanol water, 60% yield, m.p. 197–198°. A melting point of 195–196° was reported after recrystallization from ethyl acetate [20]. NMR: δ8.30–6.65 8H m, 3.95 3H s, 3.15 6H s. Molecular weight (calcd.) 283; found (MS): $m/z = 283$ (20%, M^+), 252 (4%, M^+-OCH_3), 148 (5%, M^+-$C_6H_4CO_2CH_3$), 135 (15%, $C_6H_4CO_2CH_3$), 120 [100%, $C_6H_4N(CH_3)_2$]. This compound was found identical to that previously described [21].

Animal treatment

Male Wistar rats (200–225 g) were treated with one of the following compounds: clofibrate, 300 mg/kg/day, i.p., 7 days; PB in 0.9% NaCl, 75 mg/kg/day, i.p. 4 days; β-naphthoflavone (BNF) in corn oil, 40 mg/kg/day, i.p. 3 days; isosafrole in corn oil, 150 mg/kg/day, i.p., 4 days; or pregnenolone-16α-carbonitrile suspended in 0.9% NaCl with a minimal amount of Tween 80 to provide stability, 50 mg/kg/day, p.o., 5 days. Control rats for each group received vehicle alone. Twenty-four hours after the last dose of drug, livers were homogenized in 0.15 M KCl in 10 mM Tris–HCl, pH 7.4, and microsomes were prepared by differential centrifugation.

Enzyme preparation

Partially purified rat liver cytochrome P450 (specific content = 3.0 to 3.5 nmol/mg protein) was prepared by passing solubilized microsomes through

an octylamino Agarose column. Typical recovery was 75% with a 3-fold increase in specific content of total P450. Purified NADPH–cytochrome P450 reductase was prepared by methods previously described [5, 22]. Specific activity of the reductase was 33 units/mg protein assayed using cytochrome *c* as electron acceptor.

In vitro *incubations*

Rat hepatic microsomes were incubated at 37° for 10 min using 0.08 to 0.44 mg microsomal protein, 1 mM NADP, an NADPH-generating system consisting of 5 mM glucose-6-phosphate and one unit glucose-6-phosphate dehydrogenase, 0.1 mM substrate, 10 mM magnesium chloride, 50 mM 4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.4) in a final volume of 0.5 mL. A nitrogen or carbon monoxide environment was established in side arm tubes closed with rubber septa. Gas was passed through the tubes for 10 min while shaking on ice. Unless otherwise indicated, experiments were performed in an atmosphere of nitrogen. Reactions were started by tipping in NADP from the side arm and terminated by addition of 0.3 mL of 1 M NaOH. Zero time samples contained all components, and 0.3 mL of 1 M NaOH was added prior to the addition of microsomes. Primary amines obtained from the reduction were partitioned into hexane or toluene and then into 0.1 M sodium acetate, pH 4.0, and determined fluorometrically by a previously described method [16, 23]. No significant reduction of any of the substrates was seen in the absence of either NADP or enzyme. Inhibitors of cytochrome P450 activity were added to the reaction as methanolic (piperonylbutoxide, α-naphthoflavone, metyrapone, octylamine) or aqueous (SKF 525-A) solutions in a final concentration of 0.1 mM. There were no discernible effects of methanol (2% final concentration) on the reduction rate.

In the reconstituted mixed-function oxidase system, dye reduction was measured after preincubating 0.2 nmol NADPH–cytochrome P450 reductase and 0.10 nmol cytochrome P450 (except where otherwise indicated), and 10 μg dilauryl phosphatidyl choline (DLPC) for 10 min at room temperature. The remaining incubation mixture was that described above. The rate of reduction by flavoprotein alone was determined in a separate experiment omitting the cytochrome P450. The rate of reduction of azobenzenes having electron-donating groups at the 4-position and electron-withdrawing groups at the 2′-position was linear for 10 min. Reduction of all other substrates was linear for 30 min.

Each experiment was performed in triplicate and repeated four times.

Protein was assayed by the method of Lowry *et al.* [24].

RESULTS

The chemical structures of the azo dye substrates are presented in Fig. 1. The designations, S and I, refer to the oxygen and carbon monoxide sensitivity or insensitivity of their microsomal reduction (see below). Their rates of reduction by rat liver microsomes in an anaerobic (N_2) atmosphere are indicated in Table 1. The reactions were performed at apparently saturating substrate concentrations although true saturation in most cases could not be obtained due to limited solubility of the azo dyes. Each of the insensitive substrates was readily reduced by microsomes in the presence of air or carbon monoxide (Fig. 2), whereas reduction of each of the sensitive substrates was greatly inhibited under these conditions (Fig. 3). It was of interest that the ring nitrogen in the heterocyclic analog of DAB, 4-(2-pyridylazo)-*N*,*N*-dimethylaniline (No. 7, Fig. 1, right panel), apparently altered the electron configuration so that the compound behaved as a sensitive substrate. The relative oxygen sensitivity of insensitive and sensitive substrate reduction is also seen in Fig. 4. These experiments were performed in atmospheres containing various mixtures of oxygen and nitrogen. As little as 3% oxygen completely inhibited the reduction of two sensitive substrates, *o*-methyl red and *p*-methyl red, whereas the reduction of DAB, an insensitive substrate, was unaffected by 20% oxygen. These results suggested that at least two azoreductases were involved with the two sets of substrates and that the respective mechanisms probably were not the same. That both of these systems involve cytochrome P450 was reflected in the inhibition of reduction of DAB and *o*-methyl red, representing the insensitive and sensitive substrate group, respectively, by typical inhibitors of cytochrome P450 (Fig. 5). Response of the insensitive and sensitive substrates to the inhibitors varied, although cytochrome P450 was involved in both cases.

Reduction was also studied in a reconstituted cytochrome P450 system, using highly purified NADPH–cytochrome P450 reductase and partially purified cytochrome P450 prepared from untreated rat liver microsomes. The relative rates of reduction of each of the substrates are indicated in Table 2. There was a 6-fold variation in reduction rates for the insensitive substrates, whereas reduction of sensitive substrates varied by nearly three orders of magnitude. In view of the difference in sensitivity to oxygen and CO between insensitive and sensitive substrates, it was important to determine whether reduction in the reconstituted system was catalyzed by cytochrome P450 or by NADPH–cytochrome P450 reductase alone. Reduction of each of the substrates was measured in the presence of the flavoprotein alone and in the fully reconstituted system. In Table 3, since two enzymes are being compared, reduction is simply expressed as picomoles per minute. This explains the apparant discrepancy with the rates shown in Table 2 since various amounts of cytochrome P450 were used in order to have measurable rates. However, the ratio of one cytochrome P450 to two NADPH–cytochrome P450 reductase was maintained throughout. The results show that all substrates were reduced to some extent by flavoprotein alone (Table 3). Addition of cytochrome P450 to the system increased the rate of reduction of insensitive substrates 3- to 14-fold, whereas reduction of sensitive substrates was increased only slightly. The major exception among

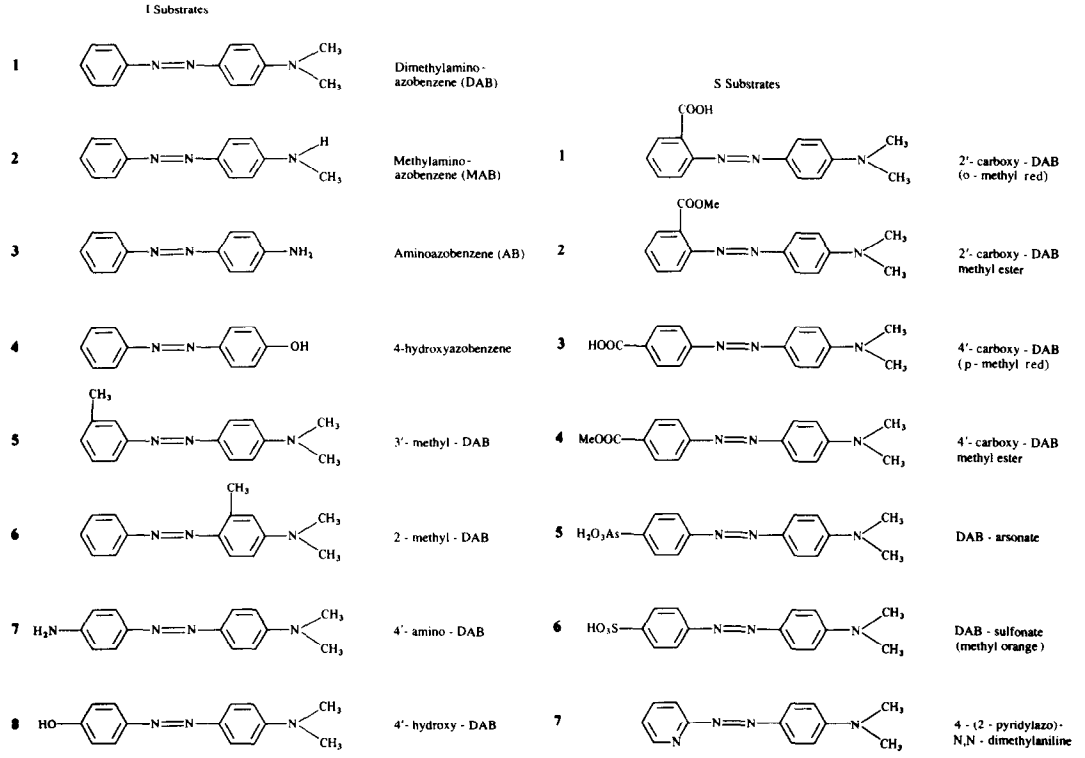

Fig. 1. Structures of azo dye substrates reduced by microsomal cytochrome P450. Left panel: I substrates, reduction of which is insensitive to oxygen and carbon monoxide. Right panel: S substrates, reduction of which is sensitive to oxygen and carbon monoxide.

Table 1. Microsomal reduction of sensitive and insensitive substrates

Substrates	Reduction* (pmol/min/mg protein)
Sensitive	
1.† 2'-Carboxy-DAB (*o*-methyl red)	21,100 ± 3500
2. 2'-Carboxy-DAB methyl ester	9200 ± 2200
3. 4'-Carboxy-DAB (*p*-methyl red)	3930 ± 290
4. 4'-Carboxy-DAB methyl ester	2030 ± 720
5. DAB-arsonate	900 ± 320
6. DAB-sulfonate (methyl orange)	1570 ± 680
7. 4-(2-Pyridylazo-*N,N*-dimethylaniline	10,700 ± 3070
Insensitive	
1. DAB	4890 ± 1080
2. MAB	9210 ± 980
3. AB	8770 ± 480
4. 4-Hydroxyazobenzene	1100 ± 180
5. 3'-Methyl-DAB	8580 ± 2000
6. 2-Methyl-DAB	3420 ± 140
7. 4'-Amino-DAB	9580 ± 880
8. 4'-Hydroxy-DAB	4350 ± 200

* Reactions were performed at apparent substrate saturation (0.1 to 0.2 mM) although limits in solubility of the dyes prevented a true assessment of saturating concentrations. Values are means ± SD, N = 4.

† The numbers correspond to those shown in Fig. 1.

the sensitive substrates was 4'-carboxy-DAB methyl ester where a 6-fold enhancement of reduction rate was seen. As seen in the microsomal system, reduction of insensitive substrates (DAB, 4'-amino-DAB, 4'-hydroxy-DAB) was still affected only slightly by CO in the reconstituted system although some sensitivity to air was seen (Fig. 6). On the other hand, reduction of sensitive substrates (*o*-methyl red, *p*-methyl red, 4'-carboxy-DAB methyl ester) in the reconstituted system was no longer CO

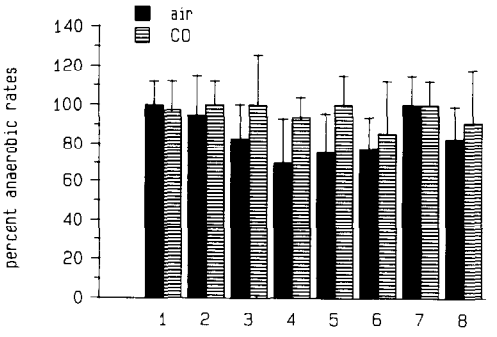

Fig. 2. Microsomal azoreduction of insensitive substrates: Effects of oxygen and carbon monoxide. Anaerobic rates were measured in an atmosphere of 100% nitrogen. Reaction tubes on ice were gassed with nitrogen or carbon monoxide or left open to the air for 10 min prior to initiating the reaction. The numbers correspond to those in Fig. 1. Anaerobic reduction rates, taken as 100%, are indicated for each substrate in Table 1. Values are means ± SD, N = 4.

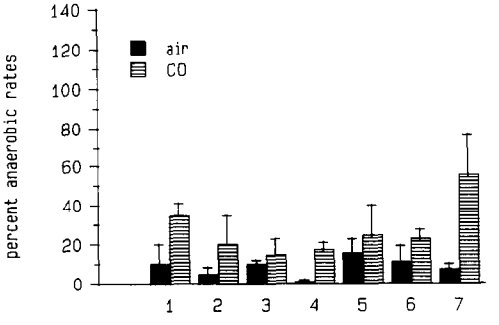

Fig. 3. Microsomal reduction of sensitive substrates: Effects of oxygen and carbon monoxide. All other conditions were as given for Fig. 2. Values are means ± SD, N = 4.

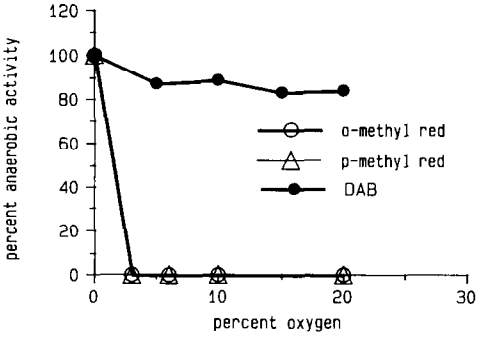

Fig. 4. Azoreduction of insensitive (DAB) and sensitive (*o*methyl red, *p*-methyl red) substrates at various oxygen concentrations. Nitrogen and air were mixed in known ratios, and the reaction tubes were gassed with these mixtures. The reactions were performed as described in Experimental Procedures. Anaerobic reduction rates, taken as 100%, are indicated for each substrate in Table 1.

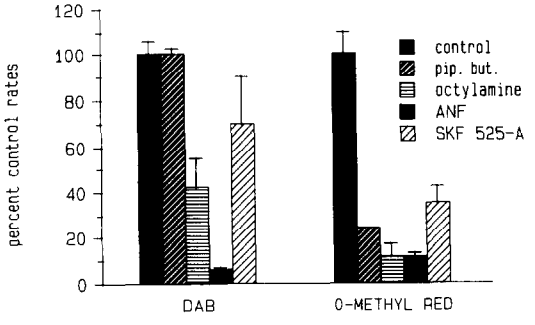

Fig. 5. Effects of inhibitors of cytochrome P450 activity on microsomal azoreduction of an insensitive substrate (DAB) and a sensitive substrate (*o*-methyl red). Inhibitors were added to the reaction tubes prior to gassing with nitrogen. Anaerobic reduction rates, taken as 100%, are indicated for each substrate in Table 1. ANF = α-naphthoflavone. Values are means ± SD, N = 4.

Table 2. Reduction of sensitive and insensitive substrates by a reconstituted cytochrome P450 system

Substrates	Reduction* (pmol/min/nmol P450)
Sensitive	
1.† 2′-Carboxy-DAB (*o*-methyl red)	63,100 ± 16,110
2. 2′-Carboxy-DAB methyl ester	15,833 ± 865
3. 4′-Carboxy-DAB (*p*-methyl red)	545 ± 221
4. 4-Carboxy-DAB methyl ester	93 ± 14
5. DAB arsonate	717 ± 104
6. DAB sulfonate (methyl orange)	2161 ± 237
Insensitive	
1. DAB	917 ± 23
2. MAB	1135 ± 133
3. AB	694 ± 110
4. *p*-Hydroxyazobenzene	224 ± 57
5. 3′-Methyl-DAB	297 ± 52
6. 2-Methyl-DAB	539 ± 203
7. 4′-Amino-DAB	187 ± 73
8. 4′-Hydroxy-DAB	595 ± 50

* Reactions were performed at apparent substrate saturation (0.1 to 0.2 mM) although limits of solubility of the dyes prevented a true assessment of saturating concentrations. Values are means ± SD, N = 4.

† The numbers correspond to those shown in Fig. 1.

Table 3. Reduction of sensitive and insensitive substrates by flavoprotein and by flavoprotein plus cytochrome P450 (reconstituted system)*

Substrates	Reduction (pmol/min) (1) Flavoprotein alone	(2) Flavoprotein + cytochrome P450	Ratio (2)/(1)
Sensitive			
1. 2′-Carboxy-DAB (*o*-methyl red)	500 ± 180	631 ± 161	1.3
2. 2′-Carboxy-DAB methyl ester	471 ± 102	633 ± 35	1.3
3. 4′-Carboxy-DAB (*p*-methyl red)	69.3 ± 0	109 ± 44	1.6
4. 4′-Carboxy-DAB methyl ester	6.1 ± 0.1	37.3 ± 5.7	6.1
5. DAB arsonate	68.9 ± 15.8	143 ± 21	2.1
6. DAB sulfonate (methyl orange)	212 ± 17	432 ± 47	2.0
Insensitive			
1. DAB	14.4 ± 3.6	202 ± 7.2	14.0
2. MAB	49.7 ± 21.0	227 ± 27	4.6
3. AB	48.0 ± 17.6	139 ± 22	2.9
4. *p*-Hydroxyazobenzene	6.0 ± 1.4	44.8 ± 11.3	7.5
5. 3′-Methyl-DAB	6.0 ± 0.0	59.3 ± 10.3	9.9
6. 2-Methyl-DAB	18.8 ± 1.9	108 ± 41	5.7
7. 4′-Amino-DAB	11.7 ± 1.7	71.6 ± 23.2	6.1
8. 4′-Hydroxy-DAB	19.0 ± 1.0	119 ± 10	6.3

* In reactions involving flavoprotein alone, all reactants of the reconstituted system were present except cytochrome P450. All other conditions were identical for the two systems. Various amounts of each of the enzymes were used, although the ratio of 2:1, reductase to cytochrome, was maintained throughout. Because two enzyme systems were compared, rates are given as pmol/min. Values are means ± SD, N = 4.

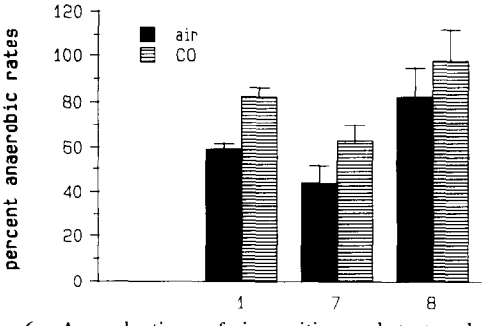

Fig. 6. Azoreduction of insensitive substrates by a reconstituted mixed-function oxidase system containing dilauroyl phosphatidylcholine, highly purified NADPH–cytochrome P450 reductase and partially purified cytochrome P450 prepared from untreated rat liver microsomes. Anaerobic rates were measured in an atmosphere of nitrogen (see Table 3). The substrate numbers refer to the structures in Fig. 1, left panel. 1 = DAB; 7 = 4′-amino-DAB; and 8 = 4′-hydroxy-DAB. Values are means ± SD, N = 4.

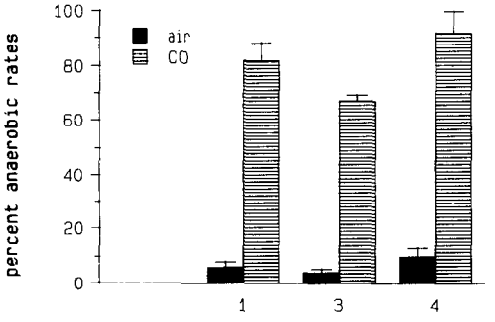

Fig. 7. Azoreduction of sensitive substrates by a reconstituted mixed-function oxidase system containing dilauroyl phosphatidylcholine, highly purified NADPH–cytochrome P450 reductase and partially purified cytochrome P450 prepared from untreated rat liver microsomes. Anaerobic rates were measured in an atmosphere of nitrogen (see Table 3). The substrate numbers refer to the structures in Fig. 1, right panel. 1 = *o*-methyl red; 3 = *p*-methyl red; and 4 = 4′-carboxy-DAB methyl ester (*p*-methyl red methyl ester). Values are means ± SD, N = 4.

sensitive although oxygen sensitivity was retained (Fig. 7). These results confirmed the principal role for cytochrome P450 in the reduction of most insensitive substrates but suggest a relatively minor role for the cytochrome in the reduction of sensitive substrates in the reconstituted system. This appeared to conflict with the microsomal studies in which reduction of sensitive substrates was inhibited markedly by CO (Fig. 3), implying a major role for cytochrome P450.

Another indication that at least two microsomal azoreductase systems were involved is seen in the responses to inducing agents (Fig. 8). Confirming previous reports from this laboratory [4], only clofibrate treatment induced the reduction of DAB and 4′-hydroxy-DAB, representative insensitive substrates, whereas reduction of *o*-methyl red, a sensitive substrate, was induced by phenobarbital, clofibrate, pregnenolone-16α-carbonitrile, isosafrole and β-naphthoflavone.

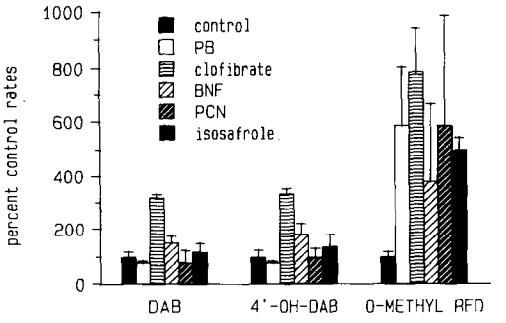

Fig. 8. Azoreduction of insensitive substrates (DAB, 4'-hydroxy-DAB) and sensitive substrate (*o*-methyl red) by microsomes prepared from rats treated with phenobarbital (PB), clofibrate, β-naphthoflavone (BNF), pregnenolone-16α-carbonitrile (PCN) or isosafrole. Anaerobic reduction rates, taken as 100%, are indicated for each substrated in Table 1. Values are means ± SD, N = 4.

DISCUSSION

Azoreduction of DAB by rat liver microsomes, by a reconstituted partially purified cytochrome P450 system and by highly purified NADPH–cytochrome P450 reductase, has been reported from this laboratory [4, 5] and elsewhere [25, 26]. Microsomal-catalyzed activity is mainly insensitive to oxygen and CO. Reduction by purified reductase is inhibited completely by oxygen, whereas reduction by the cytochrome P450 in the reconstituted system is affected only slightly by oxygen. Peisach and coworkers studied the microsomal azoreduction of amaranth and found it to be inhibited by antibodies against two major forms of cytochrome P450 and by carbon monoxide [12, 13]. They also observed considerable induction of activity by methylcholanthrene and phenobarbital and concluded that azoreductase is a general property of cytochrome P450. Martin and Kennelly reported that amaranth reduction by microsomes is oxygen insensitive [14], although other investigators concur on its oxygen sensitivity [12, 15]. We have reported previously [8] microsomal reduction of azobenzenes containing para-substituted electron-donating groups such as hydroxyl (4-hydroxyazobenzene) and amino (DAB and its corresponding secondary and primary amines). In the presence of hepatic microsomes, prominent binding spectra are observed for these substrates [8]. Azo dyes lacking such substituents (azobenzene and 4-isopropylazobenzene) exhibit no binding spectra and are not reduced by hepatic microsomes or a reconstituted cytochrome P450 system [8]. Lack of substrate reactivity is also seen for *p*-benzoylaminoazobenzene where the electron-withdrawing carbonyl function nullifies the electron-donating property of the amino group. Further differences between cytochrome P450-reducible and non-reducible dyes were revealed in attempts to synthesize their hydrazo intermediates in an anaerobic environment using metallic zinc as a two-electron reducing agent [8]. It was impossible to isolate intermediate hydrazo forms of the substrates, 4-aminoazobenzenes and 4-hydroxyazobenzene, and only fully reduced primary amines were detectable. The one exception was DAB which forms a spectrally distinct hydrazo intermediate which is exquisitely sensitive to oxygen, and even under nitrogen rapidly disproportionates to the parent azo dye and primary amine in an aqueous environment. In contrast to the cytochrome P450-reducible azo dyes, those compounds which are not substrates (4-isopropylazobenzene, 4-benzoylaminoazobenzene, azobenzene) are readily reduced to stable hydrazo intermediates by metallic zinc. It is inferred that cytochrome P450-catalyzed reduction involves enzymic donation of two electrons to bound dyes to form the hydrazo intermediate which spontaneously degrades to primary amines. Thus, azo dyes which are not reduced by microsomes apparently do not bind to enzyme and exhibit stable hydrazo intermediates. Even these intermediates are reduced extremely slowly by microsomes.

The present study confirms that both microsomes and a fully reconstituted mixed-function oxidase system containing both NADPH–cytochrome P450 reductase and cytochrome P450 readily reduce azobenzenes containing electron-donating substituents in the 4-position. Microsomal reduction of dyes having electron-donating ring substituents in other positions as well, e.g. $-CH_3$, $-NH_2$, -OH (insensitive substrates) (Fig. 1), was similarly insensitive to both oxygen and CO (Fig. 2). By contrast, microsomal reduction of substituted azobenzenes having electron-donating groups in the 4-position plus electron-withdrawing groups elsewhere on the ring system, e.g. COOH, -COOMe, $-AsO_3H_2$, $-SO_3H$ (sensitive substrates) (Fig. 1), was sensitive to both air and CO (Fig. 3). In the reconstituted system, reduction of insensitive substrates was inhibited partially by CO and oxygen sensitivity increased slightly (Fig. 7). On the other hand, although oxygen sensitivity was retained, reduction of sensitive substrates in the reconstituted system was no longer CO sensitive, implying loss of cytochrome P450 function. These results are supported by comparing reduction rates with purified NADPH–cytochrome P450 reductase alone and the fully reconstituted mixed-function oxidase system. The low activity of insensitive substrates with reductase alone was enhanced many-fold upon addition of cytochrome P450 (Table 3). This confirms that reduction is catalyzed mainly by cytochrome P450, supporting previous work in this laboratory with DAB alone [5]. With the exception of *p*-methyl red ester, rates of reduction of sensitive substrates by reductase alone were increased only slightly by addition of cytochrome P450 (Table 3), implying that the cytochrome makes little contribution in the reconstituted system. We believe that this is attributable to imperfect coupling between reductase and cytochrome in the reconstituted system.

The combination of both electron-donating and withdrawing substituents in the sensitive substrates facilitates electron delocalization throughout the molecule from the unshared pair of electrons of the electron-donating group towards the electron-withdrawing group. This is most pronounced when the groups are ortho or para to the azo linkage [27, 28]. Electron delocalization also stabilizes both one- and two-electron reduced metabolites [27].

Mason and coworkers [15] showed that the oxygen-sensitive microsomal reduction of sulfonazo III, an azo dye substituted with electron-donating and electron-withdrawing groups, produces a one-electron radical anaerobically which is sufficiently stable to be detectable by EPR and to react with oxygen upon admitting air to the system. This is analogous to our finding of oxygen sensitivity of sensitive substrates. Thus, the difference in oxygen sensitivity between sensitive and insensitive substrate reduction is based on differences in electron delocalization within the molecule and consequent formation of a relatively stable free radical. Other stable free radicals have been reported, including diphenyl picrylhydrazyl, an EPR standard [29], compounds I of catalase and horseradish peroxidase [30], a tyrosyl free radical in ribonucleotide reductase [31], and FMNH semiquinone in NADPH–cytochrome P-450 reductase [32].

It is unlikely that the differences in sensitive and insensitive substrate characteristics are due to varying lipid solubility since the octanol/water partition coefficients for the substrates did not distinguish between the two groups (data not presented). Difference in electron delocalization also predicts that the pK values of the sensitive and insensitive substrates will be different [27]. This may be the basis for the selective affinity of sensitive and insensitive substrates for different species of cytochrome P450.

Further support for two independent cytochrome P450 azoreductase systems is found in the induction experiments. Microsomal reduction of representative insensitive substrates was induced by clofibrate but not phenobarbital, β-naphthoflavone, isosafrole, or pregnenolone-16α-carbonitrile (Fig. 8), confirming previous work from this laboratory [4]. Microsomal reduction of sensitive substrates, on the other hand, was induced by several agents, suggesting that several forms of cytochrome P450 catalyze this activity. This system resembles the microsomal azoreductase described by Fujita and Peisach [12] in which the reduction of amaranth is induced by phenobarbital and methylcholanthrene and is sensitive to both oxygen and CO.

Differential sensitivity to CO raises an interesting question. If CO simply displaces oxygen from the sixth ligand position of the heme, and oxygen is not required for azoreduction, why should reduction of any azo compound be inhibited by CO? One possibility is that bound CO prevents the transfer of electrons from heme to selective substrates. At the present time this remains an unresolved matter.

In conclusion, two classes of azo reductase have been described which differ in their sensitivity to oxygen and CO, in their response to inducing agents, and in their specific requirements for electron-donating or -withdrawing substituted substrates. Both systems primarily involve selective forms of cytochrome P450. The structure–activity relationship observed will serve as the basis for further investigation of the mechanisms of reduction of azo dye carcinogens.

Acknowledgements—This research was supported by Grants CA14231 and P30CA13330 from the National Cancer Institute, National Institutes of Health.

REFERENCES

1. Weisburger JH and Williams GM, Metabolism of chemical carcinogens. In: *Cancer 1. A Comprehensive Treatise* (Ed. Becker FF), pp. 241–333. Plenum Press, New York, 1982.
2. Stoddart AM and Levine WG, Azoreduction of dimethylaminoazobenzene (DAB) in primary cultures of rat hepatocytes. Effect of hypolipidemic agents. *Drug Metab Dispos* **18**: 36–41, 1990.
3. Levine WG, Studies on microsomal azoreduction. *N,N*-Dimethyl-4-aminoazobenzene (DAB) and its derivatives. *Biochem Pharmacol* **34**: 3259–3264, 1985.
4. Raza H and Levine WG, Azoreduction of *N,N*-dimethyl-4-aminoazobenzene (DAB) by rat hepatic microsomes. Selective induction of clofibrate. *Drug Metab Dispos* **14**: 19–24, 1986.
5. Levine WG and Raza H, Mechanism of azoreduction of dimethylaminoazobenzene by rat liver NADPH–cytochrome P-450 reductase and partially purified cytochrome P-450. Oxygen and carbon monoxide sensitivity and stimulation by FAD and FMN. *Drug Metab Dispos* **16**: 441–448, 1988.
6. Ortin TC and Parker GL, The effect of hypolipidemic agents on the hepatic drug metabolizing enzyme system of the rat. Induction of cytochrome(s) P-450 with specificity toward terminal hydroxylation of lauric acid. *Drug Metab Dispos* **10**: 110–115, 1982.
7. Gibson GG, Orton TC and Tamburini PP, Cytochrome P-450 induction by clofibrate. Purification and properties of a hepatic cytochrome P-450 relatively specific for the 12- and 11-hydroxylation of dodecanoic acid (lauric acid). *Biochem J* **203**: 161–168, 1982.
8. Zbaida S, Stoddart AM and Levine WG, Studies on the mechanism of reduction of azo dye carcinogens by rat liver microsomal cytochrome P-450. *Chem Biol Interact* **69**: 61–71, 1989.
9. Mason RP, Peterson FJ and Holtzman JL, Inhibition of azoreductase by oxygen. The role of the azo anion free radical metabolite in the reduction of oxygen to superoxide. *Mol Pharmacol* **4**: 665–671, 1978.
10. Docampo R, Moreno SNJ and Mason RP, Generation of free radical metabolites and superoxide anion by the calcium indicators arsenazo III, antipyrylazo III, and murexide in rat liver microsomes. *J Biol Chem* **258**: 14920–14925, 1983.
11. Hernandez PH, Gillette JR and Mazel P, Studies on the mechanism of action of mammalian hepatic azoreductase—I. Azoreductase activity of reduced nicotinamide adenine dinucleotide phosphate-cytochrome *c* reductase. *Biochem Pharmacol* **16**: 1859–1875, 1967.
12. Fujita S and Peisach J, Liver microsomal cytochromes P-450 and azoreductase activity. *J Biol Chem* **253**: 4512–4513, 1978.
13. Fujita S, Okada Y and Peisach J, Inhibition of azoreductase activity by antibodies against cytochrome P-450 and P-448. *Biochem Biophys Res Commun* **102**: 492–498, 1981.
14. Martin CN and Kennelly JC, Rat liver microsomal azoreductase activity on four azo dyes derived from benzidine, 3,3′-dimethylbenzidine or 3,3′-dimethoxybenzidine. *Carcinogenesis* **2**: 307–312, 1981.
15. Peterson FJ, Holtzman JL, Crankshaw D and Mason RP, Two sites for azo reduction in the monooxygenase system. *Mol Pharmacol* **34**: 597–603, 1988.
16. Huang M-T, Miwa GT, Cornheim N and Lu AYH, Rat liver cytosolic azoreductase. Electron transport properties and the mechanism of dicumarol inhibition of the purified enzyme. *J Biol Chem* **254**: 11223–11227, 1979.
17. Miller JA and Miller EC, The carcinogenicity of certain

derivatives of *p*-dimethylaminoazobenzene in the rat. *J Exp Med* **87**: 139–156, 1948.
18. Vogel AI (Ed.), *A Textbook of Practical Organic Chemistry Including Qualitative Organic Analysis*, pp. 780–781. Longmans Green, London, 1956.
19. Kerber RC and Heffron PJ, Azimines. II. Reactions of nitrenes with azo compounds. *J Org Chem* **37**: 1592–1597, 1972.
20. Dipple A, Model Studies for azo dye carcinogenesis. *J Chem Soc* (Perkin Trans I): 447–449, 1972.
21. Kleps J and Prot T, Photoconductivity and electronic absorption spectra of azobenzene and its *p*-substituted derivatives. *Rocz Chem* **48**(9): 1525–1535, 1974; *Chem Abstr* **82**: 85880c, 1974.
22. Yasukochi Y and Masters BSS, Some properties of a detergent-solubilized NADPH–cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J Biol Chem* **251**: 5337–5344, 1976.
23. Van der Hoeven TH, A sensitive, fluorometric method for the assay of microsomal hydroxylase: *N*-Demethylation of *p*-chloro-*N*-methylaniline. *Anal Biochem* **77**: 523–528, 1977.
24. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
25. Elliott BM, Azoreductase activity of Sprague Dawley and Wistar-derived rats towards both carcinogenic and non-carcinogenic analogues of 4-dimethylaminophenylazobenzene (DAB). *Carcinogenesis* **5**: 1051–1055, 1984.
26. Lambooy JP and Koffman BM, 4-Dimethylaminoazobenzenes. Carcinogenicities and reductive cleavage by microsomal azo reductase. *Chem Biol Interact* **53**: 107–120, 1985.
27. Roberts JD and Caserio MC, *Basic Principles of Organic Chemistry*, 2nd Edn, pp. 1111–1116. W. A. Benjamin Inc., California, 1977.
28. Stead CV, Azo dyes. In: *The Chemistry of Synthetic Dyes* (Ed. Venkataraman K), pp. 249–301. Academic Press, New York, 1970.
29. Fieser LF and Fieser M, *Reagents for Organic Synthesis*, p. 347. John Wiley, New York, 1967.
30. Dolphin D, Forman A, Borg DC, Fajer J and Felton RH, Compounds I of catalase and horse radish peroxidase: π-cation radicals. *Proc Natl Acad Sci USA* **68**: 614–628, 1971.
31. Sjöberg B-M, Reichard P, Gräslund A and Ehrenberg A, The tyrosine free radical in ribonucleotide reductase from *Escherichia coli*. *J Biol Chem* **253**: 6863–6865, 1978.
32. Sugiyama T, Nisimoto Y, Mason HS and Loehr TM, Flavins of NADPH–cytochrome P-450 reductase: Evidence for structural alteration of flavins in their one-electron-reduced semiquinone state from resonance raman spectroscopy. *Biochemistry* **24**: 3012–3019, 1985.