STUDY ON *N*-DEMETHYLATION OF *N,N*-DIMETHYL-4-AMINOAZOBENZENE AND *N*-NITROSAMINES BY PROSTAGLANDIN H SYNTHASE

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Dedicated to Professor Josef Kostir on the occasion of his 90th birthday.

The *in vitro* enzymatic metabolism of carcinogenic *N,N*-dimethyl-4-aminoazobenzene, *N*-nitroso-*N*-methylaniline and *N*-nitroso-*N,N*-dimethylamine was investigated using ram seminal vesicle microsomal prostaglandin H synthase. Both *N*-nitrosamines are not converted by the studied enzyme. Formaldehyde is produced by the prostaglandin H synthase catalyzed reaction from *N,N*-dimethyl-4-aminoazobenzene. Arachidonic acid and hydrogen peroxide serve as cofactors for the oxidation of *N,N*-dimethyl-4-aminoazobenzene. The apparent Michaelis constant and the maximal velocity values for *N,N*-dimethyl-4-aminoazobenzene as a substrate are 64 μmol/l and 51.2 nmol HCHO/min/mg protein, respectively. In addition to formaldehyde, *N*-methyl-4-aminoazobenzene and 4-aminoazobenzene, two unknown substances are the products of the *N,N*-dimethyl-4-aminoazobenzene oxidation. The oxidation of *N,N*-dimethyl-4-aminoazobenzene catalyzed by prostaglandin H synthase is inhibited by glutathione, ascorbate and NADH. The results suggest that prostaglandin H synthase metabolizes *N,N*-dimethyl-4-aminoazobenzene through a one-electron oxidation mechanism, giving rise to free radicals.

Key words: Prostaglandin H synthase; Carcinogens; Azo dyes; *N*-Nitrosamines; *N*-Demethylation; Radicals.

The metabolism of xenobiotics is an important determinant in chemical toxicity. Cytochrome P450 monooxygenases (P450) or flavin-containing monooxygenases are supposed to be the most important enzymes for the metabolic activation of chemicals to reactive electrophiles as well as for their detoxication. However, Marnett and coworkers[1] showed in 1975 that during the oxidation of arachidonic acid to prostaglandins by prostaglandin H synthase (PHS) other chemicals were oxidized. These reactions were termed “co-oxidation” and the oxidized chemicals “cosubstrates”. More recently, PHS was suggested as an alternative enzyme for chemical metabolism, particularly in many extrahepatic tissues (*e.g.* urinary bladder, lung, kidney) that are low in P450 activity[2–4].

PHS exhibits two activities, as a cyclooxygenase and a peroxidase[4]. The cyclooxygenase catalyzes the addition of two moles of oxygen to one mole of arachidonic acid and forms a cyclic endoperoxide hydroperoxide, prostaglandin G_2. The peroxidase subsequently reduces the hydroperoxide to corresponding alcohol, prostaglandin H_2, which is then converted into various other prostanoic metabolites[4].

PHS can utilize a wide variety of reducing cofactors to provide the requisite pair of electrons involved in the reduction of prostaglandin G_2 to prostaglandin H_2 (ref.[4]). Although most of these cofactors undergo simple dehydrogenation, several compounds have oxygen inserted into their molecules[4–6]. Besides substrate oxygenation, *N*-demethylation of several xenobiotics was also determined[4,7–9].

Here, we extend the investigation to the *N*-demethylation of other potential substrates of PHS. A carcinogenic azo dye, *N*,*N*-dimethyl-4-aminoazobenzene (DAB), carcinogenic *N*-nitrosamines *N*-nitroso-*N*-methylaniline (NMA) and *N*-nitroso-*N*,*N*-dimethylamine (NDMA) were tested from the point of view of their abilities to be the reducing cosubstrates of PHS.

DAB is known to be a strong hepatocarcinogen, but cell transformation or tumor induction in other tissues due to this compound cannot be excluded[10]. NMA is a carcinogen causing tumors of lungs in mice and esophagus in rats and NDMA is a strong hepatocarcinogen[11,12]. These two *N*-nitrosamines were chosen as the examples of (i) symmetric *N*-nitrosoamines containing aliphatic chains in the molecule (NDMA) and (ii) asymmetric *N*-nitrosoamines containing both the aliphatic chain and an aromatic ring in the molecule (NMA). All three xenobiotics are oxidized by the P450-dependent reactions which lead to their activation in the tissues rich in P450s (refs[10–13]). Moreover, DAB and NMA were found to be oxidized by peroxidase (horseradish peroxidase as a model)[14–16], being activated by this enzyme to reactive intermediates binding to nucleic acids[14,16]. However, it is not known whether these compounds can be metabolized by another peroxidase, namely, mammalian PHS. To answer this question, we study the *in vitro* oxidation of these carcinogens by ram seminal vesicle microsomes, which contain PHS.

EXPERIMENTAL

Chemicals and Radiochemicals

Chemicals were obtained from the following sources: arachidonic acid, indomethacin and NADPH from Sigma Chemical Co.; ascorbate, DAB, *N*-methyl-4-aminoazobenzene (MAB) and 4-aminoazobenzene (AB) from Merck; NADH, yeast alcohol dehydrogenase and glutathione (reduced) from Boehringer; all other chemicals were reagent grade or better. NDMA and NMA were synthesized as described previously[17]. [^{14}C]DAB (0.5 mCi/mmol) was synthesized from [U-^{14}C]aniline (2.5 mCi/mmol) (Amersham International plc) and non-labelled *N*,*N*-dimethylaniline[18] and purified by column chromatography on basic alumina and by thin layer chromatography (TLC) on silica gel (Woelm). The labelled compound was stored in methanol at –17 °C.

Analytical Method

Fresh ram seminal glands were obtained from a local slaughterhouse, trimmed of excess fat and tissue, and stored at –70 °C until use. Microsomes were prepared as described previously[19] and used as a source for PHS. PHS-cyclooxygenase activity was determined by measuring the arachidonic acid-dependent oxygen uptake in a 2.0-ml chamber equipped with a Clark-type oxygen electrode. The incubation mixture contained, in 1.0 ml, 0.8 mg ram seminal vesicle microsomal protein, 50 μmol sodium phosphate (pH 7.4) and dimethyl sulfoxide (DMSO) as solvent, either alone or with different concentrations of DAB, NMA or NDMA, and was preincubated for 1 min at 37 °C. The reaction was initiated by addition of arachidonic acid (0.1 μmol) dissolved in 2 μl ethanol. The initial velocity of arachidonic acid oxidation was determined from the slope of the linear portion of the O_2 uptake curve.

Unless stated otherwise, one ml of the assay mixture for demethylation of DAB, NDMA or NMA catalyzed by ram seminal vesicle microsomal PHS contained 50 μmol sodium phosphate (pH 6.0, 7.4 or 8.4), 1–2 mg ram seminal vesicle microsomal protein, 5 μmol $MgCl_2$, 8 μmol semicarbazide and 0.05–150 nmol DAB, 0.05–15 μmol NMA or 0.05–100 μmol NDMA dissolved in DMSO (or water for NDMA), 100 nmol arachidonic acid or 100 nmol H_2O_2. Some arachidonic acid-containing reaction mixtures also contained 100 nmol indomethacin, which is known as an inhibitor of the PHS cyclooxygenase activity[4]. The mixture was incubated 30 min at 37 °C. The reaction was terminated by addition of 500 μl of 20% trichloroacetic acid. The amount of formaldehyde formed was measured as described by Nash[20].

Incubation mixture for study of the [^{14}C]DAB oxidation by the PHS system contained in a final volume of 1.0 ml: 50 μmol sodium phosphate (pH 6.0), 1–2 mg ram seminal vesicle microsomal protein, 5 μmol $MgCl_2$, 100 nmol arachidonic acid (or 100 nmol H_2O_2) and 100 nmol [^{14}C]DAB dissolved in DMSO (20 μl/1 ml incubation). The mixture was incubated at 37 °C (60 min) in open test tubes, after which the [^{14}C]DAB products were extracted twice with ethyl acetate (2 × 2 ml). The extracts of products and residual [^{14}C]DAB were evaporated, dissolved in a minimal volume of methanol and chromatographed on thin layers of silica gel using diethyl ether–hexane (3 : 1, v/v) for elution. The coloured and/or UV-absorbing products as well as the residual [^{14}C]DAB were mechanically separated from the layers and placed in scintillation vials; the radioactivity was counted in a Packard Ultra Gold X liquid scintillation cocktail on a Packard Tri-Carb 2000 CA scintillation counter. Identical TLC was carried out with standards (MAB and AB).

Inhibition of [^{14}C]DAB oxidation by glutathione, ascorbate and NADH was performed by the addition of 0.5 mM or 1 mM glutathione or ascorbate or NADH dissolved in 50 mM phosphate buffer (pH 6.0).

Protein concentrations were estimated according to Bradford[21] or Lowry *et al.*[22] with bovine serum albumin as a standard.

Kinetic analyses were carried out using the non-linear least-squares method described previously[23].

RESULTS

Conversion of arachidonic acid requires the incorporation of molecular oxygen catalyzed by PHS cyclooxygenase and the presence of a reducing cofactor for the reduction of endogeneous substrates (prostaglandins) catalyzed by the hydroperoxidase activity of the enzyme[7]. Oxygen uptake serves as a means of measuring cyclooxygenase activity of PHS (refs[7,24]). We investigated three carcinogenic, structurally diverse chemicals

(DAB, NMA and NDMA) as cofactors for PHS by measuring oxygen incorporation into arachidonic acid catalyzed by ram seminal microsomes containing PHS. Table I shows the concentration-dependent stimulation of oxygen uptake produced by DAB, which is completely inhibited by indomethacin, a well-known PHS cyclooxygenase inhibitor. At high concentrations of DAB, the stimulating effect of this compound was descreased (Table I). These findings indicated that DAB serves as a cosubstrate for PHS. *N*-Nitrosamines were ineffective with respect to the stimulation of the oxygen uptake.

DAB and *N*-nitrosamines are oxidized by α-*C*-hydroxylation (*N*-demethylation) by P450-catalyzed reaction[10–12]. Here, we examine whether *N*-demethylation of these compounds is also catalyzed by PHS.

The results shown in Table II indicate that DAB is demethylated in the presence of PHS and arachidonic acid to produce formaldehyde. The demethylation of DAB by PHS was measured at pH 6.0, 7.4 and 8.4. The highest rate of demethylation was detected at pH 6.0 (Table II). The ability of several structurally diverse compounds to catalyze the PHS-mediated *N*-demethylation of DAB was examined (Table III). The highest rates of DAB demethylation were obtained using H_2O_2, followed by arachidonic acid. The NADPH, which serves as cofactor for cytochrome P450-dependent *N*-demethylation of DAB in the mixed function oxidase system[10] was not effective. Similarly, cumene hydroperoxide, which can replace NADPH in P450-dependent *N*-demethylation reactions, was not effective, either (Table III). This indicates that PHS present in ram seminal vesicle microsomes is really responsible for DAB *N*-demethyla-

TABLE I

Stimulation of PHS-cyclooxygenase activity (measured as oxygen uptake rate per protein content, *v*) by various DAB concentrations (*c*). The numbers are averages and standard deviations of triplicate analyses. For experimental conditions, see the text

c, μmol/l	*v*, mol/min kg
0	105.0 ± 9.6
7.5	156.8 ± 10.3
15.0	165.0 ± 11.0
30.0	203.5 ± 14.8
100.0	169.5 ± 11.3
150.0	161.3 ± 11.0
30.0[a]	8.8 ± 0.9

[a] 100 nmol of indomethacin was added.

tion determined in our experiments. This is also confirmed by the inhibition of DAB demethylation by indomethacin (Table II).

In contrast to DAB, *N*-demethylation of both *N*-nitrosamines (NMA, NDMA) was not catalyzed by PHS fortified with arachidonic acid (Table II) or H_2O_2 (results not shown). Although attempts were made to optimize incubation conditions, demethylation did not occur with either of these nitrosamines. At pH 6.0, the apparent Michaelis constant (K_m) value and the maximal velocity (V_{max}) value of the DAB oxidative reaction for formaldehyde formation are 64 μmol/l and 51.2 nmol/min/mg protein, respectively.

Special emphasis was laid on the identification and quantitation of not only formaldehyde, but also of other metabolites formed by PHS. The radioactive [^{14}C]DAB was

TABLE II

N-Demethylation of xenobiotics by ram seminal vesicle microsomal PHS. The values are averages and standard deviations of three parallel experiments. For experimental conditions, see the text

Compound	Rate of *N*-demethylation, nmol HCHO/min/mg protein		
	pH 6.0	pH 7.4	pH 8.4
DAB (100 μM)	32.4 ± 3.0	28.7 ± 2.6	15.6 ± 1.6
+ 100 μM indomethacin	2.1 ± 0.2	1.5 ± 0.1	0.7 ± 0.1
NMA (15 mM)	[a]	[a]	[a]
NDMA (15 mM)	[a]	[a]	[a]

[a] Not detectable.

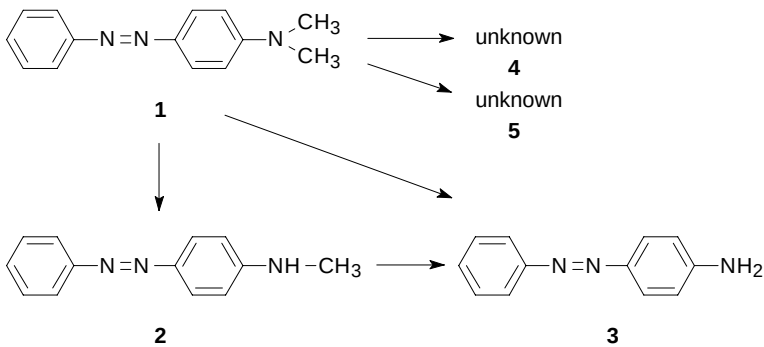
TABLE III

N-Demethylation of DAB by PHS using various cofactors. The values are averages and standard deviations of three parallel experiments. For experimental conditions, see the text

Cofactor	Rate of *N*-demethylation, nmol/min/mg protein
None	0
Arachidonic acid (0.1 mM)	32.4 ± 3.0
Hydrogen peroxide (0.1 mM)	35.1 ± 3.1
Cumene hydroperoxide (0.1 mM)	0
NADPH (2 mM)	0

used in these experiments. The conversion of [^{14}C]DAB was quantified by measuring the amounts of parent compound recovered from the complete incubation mixture (containing microsomes, [^{14}C]DAB, arachidonic acid) and comparing them with those found in control incubation (without microsomes or arachidonic acid). More than 45% of DAB is oxidized by ram seminal vesicle microsomal PHS in the presence of arachidonic acid under the conditions used (Table IV). Hydrogen peroxide is also effective for the PHS-mediated oxidation of this compound (Table IV).

The products of DAB (**1**) conversion generated by PHS in the presence of arachidonic acid were separated by TLC on silica gel. [^{14}C]DAB is oxidized by PHS to four products. Two of them cochromatographed with MAB (**2**) and AB (**3**), *i.e.* products formed by *N*-demethylation of DAB (Table IV, Scheme 1). The identity of a further two products has not yet been established.



SCHEME 1

The conversion of DAB is inhibited by glutathione, ascorbate and NADH (Table V).

Almost no products of [^{14}C]DAB were detected when 1 mM glutathione or ascorbate were present in the incubation mixture. Two mechanisms are established for the explanation of glutathione and ascorbate inhibition. Glutathione and ascorbate serve as cosubstrates for PHS peroxidase. They are oxidized to radicals[25] and hence they can compete with DAB, causing lowering of DAB oxidation. The second explanation is the following: the peroxidase first oxidizes DAB which may be a more efficient reducing cosubstrate than glutathione or ascorbate. The DAB radical produced in this oxidation is then reduced by glutathione or ascorabte forming both glutathione or ascorbate radicals and the parent DAB.

Similarly, very low amounts of products of the DAB conversion were detected when 1 mM NADH was present in the incubation medium. NADH is not converted by PHS as its cosubstrate under the conditions used. We, however, found that this compound acts as an inhibitor of the cyclooxygenase activity of PHS. It decreases the cyclooxygenase-mediated oxygen incorporation into the arachidonic acid (to 7% of the control).

Radicals formed from arachidonic acid in the cyclooxygenase activity[4] (the C-11 or the C-15 radicals of arachidonic acid) may be reduced by NADH back to the parent compound. Reduced radicals are subsequently not able to consume oxygen molecule to

TABLE IV
Products formed from [^{14}C]DAB by the ram seminal microsomal PHS expressed in the relative radioactivity (%). For experimental conditions, see the text

Product[a] R_F	Total radioactivity[b], %				
	without PHS, with arachidonic acid	without PHS or arachidonic acid	with PHS and arachidonic acid	with PHS, without arachidonic acid	with PHS and H_2O_2
5 (0.01)	1.3 ± 0.1	0.2 ± 0.01	20.7 ± 2.0	5.4 ± 0.5	21.2 ± 2.2
4 (0.28)	[c]	[c]	3.1 ± 0.3	[c]	3.2 ± 0.3
3 (0.70)	[c]	[c]	4.0 ± 0.4	0.5 ± 0.04	4.2 ± 0.4
2 (0.75)	[c]	[c]	17.8 ± 2.0	2.2 ± 0.3	18.1 ± 1.9
1 (0.8)	98.7 ± 0.9	99.8 ± 1.0	54.4 ± 5.3	91.9 ± 1.1	53.3 ± 5.2

[a] Compounds separated by TLC [diethyl ether–hexane (3 : 1, v/v)] after extraction with ethyl acetate (see Experimental). [b] Means and standard deviations of three experiments. [c] Not detected.

TABLE V
The effect of ascorbate, glutathione and NADH on the DAB demethylation catalyzed by PHS. The values are averages and standard deviations of three parallel experiments. For experimental details, see the text

Compound	Degree of DAB conversion, %
None	45.6 ± 4.3
Glutathione	
0.5 mM	25.2 ± 2.1
1.0 mM	3.5 ± 0.6
Ascorbate	
0.5 mM	19.2 ± 2.0
1.0 mM	3.1 ± 0.3
NADH	
0.5 mM	20.4 ± 1.9
1.0 mM	8.5 ± 0.8

form a cyclic 9,11-endoperoxide (from a C-11 radical) or a peroxy radical which then abstracts hydrogen to form prostaglandin G_2 (from a C-15 radical). Second explanation of the inhibition of the [^{14}C]DAB oxidation by NADH is the same as that described for glutathione and ascorbate. The primarily formed DAB radical is reduced by NADH to parent DAB and NADH is simultaneously oxidized to NAD^+. Indeed, we determined that NAD^+ is formed during the reaction. When most of the NADH (in the reaction mixture) was oxidized, the reaction adjusted to pH 8.8, and an NADH-generating system (alcohol dehydrogenase and ethyl alcohol) was added, NADH was fully reformed, indicating that the product was NAD^+.

DISCUSSION

Our results show that of the three *N*-methyl compounds tested, only DAB is enzymatically cleaved to HCHO and its corresponding demethylated derivatives (MAB, AB) in a system containing PHS. NMA or NDMA are not substrates of this enzyme. These three carcinogens are effectively demethylated by P450 monooxygenase-dependent systems. DAB is preferentially demethylated by isoenzymes P450 1A1 or 1A2 (ref.[26]), NMA by P450 2B1 or 2B2 isoenzymes[27,28] and NDMA by the isoenzyme P450 2E1 (ref.[29]) and/or P450 2B1 or 2B2 isoenzymes[27]. Other P450 isoenzymes are also effective in their metabolism, but with lower efficiencies.

It is known that the PHS-dependent system did not exhibit the broad substrate specificity reported for the P450-dependent system with regard to dealkylation[7]. This difference in substrate specificity may be a result of different dealkylation mechanisms prevalent in each case and/or the arrangement of the substrate binding site. Unfortunately, neither the nature of the active site of PHS nor the nature and number of binding sites for peroxides and cosubstrates of PHS have been elucidated[4]. The ability of a given compound to serve as a cosubstrate of PHS was found to be dependent on its lipid solubility (partition coefficient) as well as on its redox potential. It was postulated by Eling and co-workers[4,30] that in general, any lipophilic compound with a relatively low oxidation potential (*i.e.* easily oxidized) will function as a cosubstrate of PHS. Our results are consistent with these findings. The lipophilicity of the carcinogens tested is highest for DAB and decreases in the order: DAB > NMA > NDMA, DAB was the only substrate converted.

The products of the DAB oxidation formed by PHS are identical with those formed by another peroxidase (horseradish peroxidase)[16] as well as with those formed by P450-dependent system[10]. The mechanism of the peroxidase reaction is not yet clear. It is known that *N*-dealkylation of several substrates by PHS (or horseradish peroxidase) is different from the *N*-dealkylation mechanism suggested for P450 (refs[7,31–34]). *N*-Dealkylation catalyzed by peroxidases results in the formation of a free cation radical and iminium cation by sequential one-electron oxidations, the latter of which being hydrolyzed to formaldehyde and the demethylated amine[7,31–34]. We found that DAB is oxidized by

PHS (or horseradish peroxidase) to reactive metabolites, which covalently bind to nucleic acids[14] and this binding is inhibited by compounds which also act as radical scavengers (glutathione, ascorbate, *etc*.)[14]. Moreover, the metabolites formed by PHS (present paper) or horseradish peroxidase[14] also effectively oxidize NADH. Free radicals should thus be formed as the primary products. Further experiments are, however, needed to confirm the above mentioned mechanism suggested for *N*-dealkylation of other substrates by PHS (refs[7,31–34]).

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