Toxicology Letters, 70 (1994) 147–153

TOXLET 02971

Comparison of aniline hydroxylation by hemoglobin and microsomal cytochrome P450 using stable isotopes

Hugh A. Barton and Michael A. Marletta[1]

Program in Toxicology, Massachusetts Institute of Technology, Cambridge, MA (USA)

(Received 5 January 1993)
(Accepted 7 March 1993)

Key words: Aniline; Hemoglobin; Cytochrome P450

SUMMARY

Hemoglobin (Hb) and cytochrome P450 carry out aromatic ring hydroxylation of aniline. In the presence of reductants and Hb, *para*- and *ortho*-aminophenol were formed. Under $[^{18}O]O_2$, 100% of product was labeled; no incorporation occurred with $[^{18}O]H_2O$. Deuterium (1.9%) was detectable in *p*-aminophenol formed from *p*-[^{2}H]aniline by Hb, as compared with 6% retention observed with cytochrome P450. These observations are consistent with a mechanism for Hb-dependent reaction involving formation of an iron–oxo complex competent to hydroxylate substrate. Hb-mediated reactions may represent a source of extrahepatic metabolism since Hb is a major carrier for small organic molecules. The similarities of P450- and Hb-mediated aniline hydroxylation using stable isotopes preclude their use as in vivo probes.

INTRODUCTION

The ability of cytochromes P450, particularly P4502E1, to catalyze hydroxylations and other reactions with small organic molecules has been intensively studied [1,2]. Hemoglobin (Hb) and myoglobin have been found to carry out some similar reactions with a variety of substrates [3,4]. Aromatic and aliphatic hydroxylations, N-dealkylation, and O-dealkylation have been demonstrated using Hb and a variety of

Correspondence to: Dr. Hugh A. Barton, ManTech Environmental Technology Inc., P.O. Box 31009, Dayton, OH 45437-009, USA.

[1]Present address: College of Pharmacy, University of Michigan, Ann Arbor, MI, 48109, USA.

reducing systems (e.g., ascorbate, dihydroxyfumarate, P450 reductase/NADPH) [3–5].

Heme proteins are classified into four categories (oxygen carriers, peroxidases, dioxygenases, and monooxygenases) based on their predominant function in vivo. However, proteins from each group have some ability to carry out monooxygenase-like chemistry in the presence of reductants and O_2 [6]. The heme-pocket active site structures and implications for their enzymatic activities have been compared for four heme-enzymes [6].

The study described here extends the characterization of the Hb-mediated hydroxylation of aniline [7]. Possible in vivo significance of such reactions is further suggested by the finding that Hb acts as the major blood carrier for small organic molecules including hexane and toluene [8]. These experiments were undertaken to determine whether stable isotope studies might be a useful mechanistic probe for the occurrence in vivo of Hb- and P450-mediated aniline hydroxylation.

MATERIALS AND METHODS

Materials

Methemoglobin (human type III) and L-ascorbic acid (sodium salt) were obtained from Sigma (St. Louis, MO). $[^{18}O]O_2$, $[^{18}O]H_2O$, and deuterium gas were purchased from KOR Isotopes (Cambridge, MA). Other chemicals were obtained as follows: palladium (5%) on activated charcoal, $[^2H]CH_3OH$ (99%), and the aminophenol standards, Aldrich (Milwaukee, WI); *p*-bromoaniline, MCB (Cincinnati, OH). All other solvents or chemicals were at least reagent grade. Aniline was distilled and stored under argon before use. Microsomes and purified P450 reductase were a generous gift of Dr. Rosemary L. Sousa. Liver microsomes (2.9 nmol P450/mg protein) were prepared from male Long Evans rats pretreated with phenobarbital. Purified P450 reductase was determined to be homogeneous by SDS gel electrophoresis and it reduced cytochrome *c* at a rate of 27.4 μmol/min/mg (1 unit = 1 μmol/min/mg).

Synthesis of p-[^{2}H]aniline

p-Bromoaniline was recrystallized from ethanol/water to remove the contaminating aniline (about 5% by GC-MS). The exchangeable hydrogens were replaced with deuterium by dissolving the chemical in excess $[^2H]CH_3OH$ followed by solvent removal under vacuum. In a hydrogenator, 0.19 g (90 μmol) 5% palladium on charcoal was charged with deuterium at 6 psi. Then, 1 g (5.8 mmol) *p*-bromoaniline dissolved in 10 ml ethyl acetate and 1.6 ml (11.5 mmol) triethylamine were added to the palladium. The reaction was stirred for 5 h with frequent adjustment of the deuterium pressure to 6 psi. At the end of the 5 h, GC-MS analysis showed less than 0.5% *p*-bromoaniline remaining. The reaction mixture was shaken with an equal volume of H_2O to reexchange the amine deuteriums. Ethyl acetate was removed, the water reextracted with ethyl acetate, and the combined organic fractions dried with $MgSO_4$. The dried solution was evaporated in vacuo and the resulting liquid was distilled in a

microdistillation apparatus giving 0.25 g (2.7 mmol) product (47% yield). The product was determined to be pure by GC-MS analysis using both electron impact (EI) and chemical ionization (CI). The product was labeled exclusively in the *para* position as determined by NMR (Bruker WM250) using $CDCl_3$. Shifts are reported relative to residual chloroform as standard at 7.24 ppm: 3.63 (2H, *s*, $-NH_2$); 6.68 (2H, *d*, J = 8 Hz); 7.15 (2H, *d*, J = 8 Hz).

Hemoglobin incubations

Incubations were carried out in acid-washed glassware in phosphate buffer with low iron content. These precautions reduced the metal-catalyzed, ascorbate-dependent background hydroxylation reaction. Reaction solutions contained 10 μM methemoglobin, 1 mM ascorbate, and 40 mM aniline (or *p*-[^{2}H]aniline) in 1 ml final volume of 100 mM potassium phosphate buffer (pH 7.0). Alternatively, ascorbate was replaced by 250 μM NADPH and 0.022 units P450 reductase. Reactions were carried out for 30 or 60 min at 37°C. [^{18}O]O_2 incubations were carried out as described above for the Hb reactions. Incubations were purged extensively with argon following which the labeled gas was added.

Assay for aminophenols and 4-acetamidophenol by GC-MS

Reactions were terminated by addition of 40 μl of a 28% (w/v) $ZnSO_4$ solution and centrifuged for 3 min at 1300 × *g*. An aliquot (0.8 ml) of the supernatant was removed. Subsequent to the determination that *meta*-aminophenol was not formed in the incubations, 1 μl of a 5 mM solution in ethanol was added as an internal standard before extraction with $CHCl_3$ (3 × 0.8 ml) to remove the aniline. The aqueous solution was added to 100 ml H_2O and derivatized with acetic anhydride and trifluoroacetic anhydride [9]. The resulting product was unstable and was analyzed immediately. A Hewlett Packard 5887A Gas Chromatograph-Mass Spectrometer system was used in the EI and CI mode with either a Hewlett Packard 20 m methyl silicone column or a DB-5 30 m column from J & W Scientific (Cordoba, CA). Analysis was done using selected ion monitoring for several major ions: M (*m/z* 136, 187, 205, 247) and M + 2 (*m/z* 138, 189, 207, 249) were analyzed for the ^{18}O labeling; M, M − 1, M + 1, M + 2 were analyzed for the ^{2}H labeling experiments. Peaks were integrated for each ion and the areas compared. Alternatively, samples were acetylated only and analyzed for M (EI: 109, 151, 193; CI: 194, 222), M − 1, M + 1, M + 2. The percent of product labeled with ^{18}O was normalized using the atom-percent excess determined in triplicate for 4-acetamidophenol formed by P450-catalyzed hydroxylation of acetanilide. This reaction has been shown to incorporate the oxygen solely from molecular oxygen [10]. Incubations with acetanilide were carried out identically to, and in some cases simultaneously with, P450 or Hb reactions with aniline. Standards used for quantifying the amounts of products were added to 0.8 ml buffer, extracted, and derivatized.

RESULTS

Products of hemoglobin and microsomal P450 reactions

Reactions carried out for 30 min with methemoglobin, ascorbate, and aniline produced *p*-aminophenol and *o*-aminophenol as determined by the identical retention times and mass spectra of the reaction products and authentic standards (Table I). Both isomers were also found in reactions with NADPH/P450 reductase/aniline/methemoglobin or NADPH/aniline/methemoglobin. Control incubations with ascorbate and aniline also formed the two aminophenol isomers. Presumably, this ascorbate background reaction was catalyzed by contaminating iron or other metals since it was reduced by acid washing glassware. No *m*-aminophenol was detected, nor was product detected in the absence of Hb when NADPH and P450 reductase was the reductant or in the presence of Hb without reductant. Similar results were obtained using oxyhemoglobin, but for ease of performing the $[^{18}O]O_2$ experiments methemoglobin was used.

The product ratio in ascorbate-only background reactions was 0.5 (*para*/*ortho*). The Hb-mediated reaction, using either reducing system, formed the *para* isomer preferentially. Hb/ascorbate (net reaction above the ascorbate background) showed a ratio of 5. Preference for the *para* isomer (ratio of 8) was found with Hb/NADPH/P450 reductase.

Both *ortho* and *para* isomers were formed in reactions containing microsomal P450; no *meta* was detectable. A product ratio of 1.1 was found with rat liver microsomes.

TABLE I

AMINOPHENOL ISOMERS FORMED

System	*p*-AP[a]	*m*-AP[a]	*o*-AP[a]	*p*-AP/*o*-AP
Hb/ascorbate	11.0 ± 4.0	0	2.0 ± 1.0	5.0
Hb/NADPH/P450 reductase	2.5 ± 0.3	0	0.33 ± 0.01	8.0
Ascorbate	0.6 ± 0.01	0	1.3 ± 0.3	0.5
P450 (microsomes)/ NADPH	9.1 ± 1.0	0	10.0 ± 1.8	1.1
P450 reductase/ NADPH	0	0	0	0

[a] nmol formed in 30 min incubation.

Incubations with ^{18}O

Reactions in $[^{18}O]O_2$ atmosphere using Hb with ascorbate as reductant showed 100% labeling ($n = 6$) of each isomer (Table II). Incorporation in the ascorbate control incubations was also 100%. Incorporation of ^{18}O was identical in the hydroxylation of aniline and acetanilide catalyzed by microsomal P450, indicative of 100% incorporation. To confirm that O_2 was the only source of oxygen, the reactions were also carried out in $[^{18}O]H_2O$. Reactions run in 90% $[^{18}O]H_2O$ showed no increase over the normal 1–2% background of M + 2 ions. NADPH/P450 reductase with Hb in 50% $[^{18}O]H_2O$ also showed no label incorporation.

Incubations with p-[^2H]aniline

Retention of deuterium in *para*-hydroxylated products, often referred to as the NIH shift, is characteristic of P450-mediated aryl hydroxylation but not of ascorbic acid/Fe(II)-catalyzed reactions in aqueous solution [14]. *o*-Aminophenol formed in the reactions with *p*-[^{2}H]aniline was essentially 100% deuterium labeled as expected (Table II). *p*-Aminophenol showed only 1.9% ± 0.4% ($n = 3$) retention of deuterium in methemoglobin-containing incubations and in ascorbate only controls. The identical low retention was seen when P450 reductase and NADPH replaced ascorbate. Rat liver microsomal P450-catalyzed hydroxylation showed 5.6 ± 0.5% ($n = 2$) retention. This is virtually identical to the 6% retention reported for rabbit liver microsomes [13].

TABLE II

INCORPORATION OF ISOTOPIC LABEL INTO THE AMINOPHENOL PRODUCTS FORMED FROM ANILINE

System	% product labeled[a]		
	ortho	*para*	*(n)*
Hemoglobin[c]/ascorbate/$[^{18}O]O_2$	100	100	(6)
Hemoglobin/ascorbate/$[^{18}O]H_2O$	ND[b]	ND	(2)
Hemoglobin/P450 reductase/NADPH/$[^{18}O]H_2O$	ND	ND	(2)
Ascorbate/$[^{18}O]O_2$	100	100	(1)
P450 (microsomes)/P450 reductase/NADPH/$[^{18}O]O_2$	100	100	(3)
Hemoglobin[c]/ascorbate/[^{2}H]aniline	100	2	(3)
Hemoglobin/P450 reductase/NADPH/[^{2}H]aniline	100	2	(2)
Ascorbate/[^{2}H]aniline	100	2	(2)
P450 (microsomes)/P450 reductase/NADPH/[^{2}H]aniline	100	6	(2)

[a] % product labeled = [labeled/(labeled + unlabeled)] × 100.

[b] ND, not detected (≤ 3% incorporation detected).

[c] Hb was added as methemoglobin.

DISCUSSION

Analysis of all the aminophenol isomers resulting from aniline hydroxylation consistently shows the formation of only *ortho*- and *para*-aminophenol. *m*-Aminophenol has never been detected in any of the numerous aniline hydroxylating systems due to the *ortho-para*-directing nature of the amino substituent in electrophilic aromatic substitution reactions. The ratio of *para* to *ortho* is, however, quite varied in different systems. As exemplified in the work reported here, there is a large reversal in regiospecific hydroxylation in the ascorbate background (*p/o* = 0.5) and Hb-dependent (*p/o* = 5) reactions. The shift in regiospecificity suggests that there are steric factors imposed by the protein. This would be expected if the reactive oxygen species responsible for hydroxylation is formed in an environment that constrains the geometry of aniline binding and reaction. Such effects, of course, are commonplace with enzymes and could be expected for other proteins such as Hb when the reaction occurs within the heme-pocket.

Ratios of product formed in vitro with liver microsomes from several species are variable (*p/o* = 1–6), although always equal to or greater than 1.0 [11,12]. In vivo exposures of rats and mice also produced urinary metabolites derived predominantly from *p*-aminophenol, but again there was a large interspecies variation (rats: 8.1, mice: 1.6). Overall, the Hb- and P450-mediated results are similar but contrast with the ascorbate/iron background reaction.

The ^{18}O experiments demonstrate that Hb, like P450, activates molecular oxygen for incorporation into the parent compound in the presence of either reducing system. In addition, virtually no product was produced when the incubations were saturated with argon and O_2 excluded. The lack of incorporation of ^{18}O from $[^{18}O]H_2O$ under $[^{16}O]O_2$ atmosphere indicates that product does not result from mechanisms that produce either free hydroxyl radicals or aniline cation radicals in solution. Rather, it appears that an activated oxygen–iron complex is formed by both proteins.

Retention of deuterium varies greatly depending upon the substrate; aniline shows low levels of retention as compared to others [13]. The Hb-mediated reaction shows lower retention than the P450-catalyzed hydroxylation. This may indicate mechanistic differences or differences in the degree of aprotic active site environment. Castle and Lindsay-Smith [14] have shown that deuterium retention during the hydroxylation of anisole and acetanilide by hydroxyl radicals occurs to as high a degree as that found with P450 when aprotic solvents were used, although there was no retention in aqueous solutions.

Hb-mediated reactions are of interest for mechanistic comparisons with P450 and their potential pharmacological or toxicological significance [15]. The ability of Hb to carry out monooxygenase reactions in the presence of the serum constituent ascorbate or the methemoglobin-reducing enzyme system in the red blood cells (Magee, B.H. and Marletta, M.A., unpublished results) may be important for in vivo oxidation of therapeutic drugs or environmental toxins. The studies reported here show the two protein-mediated reactions share mechanistic similarities. The differences are too

small to allow the use of these stable isotopes as probes for distinguishing the two reactions in vivo.

ACKNOWLEDGEMENT

We wish to thank Dr. John S. Wishnok for invaluable assistance and advice in obtaining the mass spectral data.

REFERENCES

1 Morgan, E.T., Koop, D.R. and Coon, M.J. (1982) Catalytic activity of cytochrome P-450 isozyme 3a isolated from liver microsomes of ethanol-treated rabbits: Oxidation of alcohols. J. Biol. Chem. 257, 13951–13957.
2 Guengerich, F.P., Kim, D.-H. and Iwasaki, M. (1991) Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspect. Chem. Res. Toxicol. 4, 168–179.
3 Starke, D.W., Blisard, K.S. and Mieyal, J.J. (1984) Substrate specificity of the monooxygenase activity of hemoglobin. Mol Pharmacol. 25, 467–475.
4 Starke, D.W. and Mieyal, J.J. (1989) Hemoglobin catalysis of a monooxygenase-like aliphatic hydroxylation reaction. Biochem. Pharmacol. 38, 201–204.
5 Guillochon, D., Cambou, B., Esclade, L. and Thomas, D. (1988) Ascorbic acid and dihydroxyfumaric acid dependent hydroxylase activity of immobilized haemoglobin. Enzymol. Microbiol. Technol. 6, 161–164.
6 Dawson, J.H. (1988) Probing structure-function relations in heme-containing oxygenases and peroxidases. Science 240, 433–439.
7 Mieyal, J.J., Ackerman, R.S., Blumer, J.L. and Freeman, L.S. (1976) Characterization of enzyme-like activity of human hemoglobin. J. Biol. Chem. 251, 3436–3441.
8 Lam, C.-W., Galen, T.J., Boyd, F.J. and Pierson, D.L. (1990) Mechanism of transport and distribution of organic solvents in blood. Toxicol. Appl. Pharmacol. 104, 117–129.
9 Coutts, R.T., Hargesheimer, E.E., Pasutto, F.M. and Baker, G.B. (1981) Analysis of aniline and aminophenols in aqueous solutions using electron capture gas chromatography. J. Chromatog. Sci. 19, 151–155.
10 Psner, H.S., Mitoma, C., Rothberg, S. and Udenfriend, S. (1961) Enzymic hydroxylation of aromatic compounds. Arch. Biochem. Biophys. 94, 280–290.
11 Radzik, D.M. and Kissinger, P.T. (1984) Determination of aniline and metabolites produced in vitro by liquid chromatography/electrochemistry. Anal. Biochem. 140, 74–83.
12 Posner, H.S., Mitoma, C. and Udenfriend, S. (1961) Enzymatic hydroxylation of aromatic compounds. II. Further studies of the properties of the microsomal hydroxylase system. Arch. Biochem. Biophys. 94, 269–279.
13 Daly, J., Jerina, D. and Witkop, B. (1968) Migration of deuterium during hydroxylation of aromatic substrates by liver microsomes: I. Influence of ring substituents. Arch. Biochem. Biophys. 128, 517–527.
14 Castle, L. and Lindsay-Smith, J.R. (1980) Aromatic hydroxylation by oxy-radicals–a possible model for hydroxylation catalysed by cytochrome P-450. J. Mol. Catal. 7, 235–243.
15 Juchau, M.R. and Symms, K.G. (1972) Aniline hydroxylation in the human placenta–mechanistic aspects. Biochem. Pharmacol. 21, 2053–2064.