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Role of Human Liver P450s and Cytochrome b_5 in the Reductive Metabolism of 3′-Azido-3′-deoxythymidine (AZT) to 3′-Amino-3′-deoxythymidine

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ABSTRACT. Our laboratory has shown that human liver microsomes metabolize the anti-HIV drug 3′-azido-3′-deoxythymidine (AZT) via a P450-type reductive reaction to a toxic metabolite 3′-amino-3′-deoxythymidine (AMT). In the present study, we examined the role of specific human P450s and other microsomal enzymes in AZT reduction. Under anaerobic conditions in the presence of NADPH, human liver microsomes converted AZT to AMT with kinetics indicative of two enzymatic components, one with a low K_m (58–74 μM) and V_{max} (107–142 pmol AMT formed/min/mg protein) and the other with a high K_m (4.33–5.88 mM) and V_{max} (1804–2607 pmol AMT formed/min/mg). Involvement of a specific P450 enzyme in AZT reduction was not detected by using human P450 substrates and inhibitors. Antibodies to human CYP2E1, CYP3A4, CYP2C8, CYP2C9, CYP2C19, and CYP2A6 were also without effect on this reaction. NADH was as effective as NADPH in promoting microsomal AZT reduction, raising the possibility of cytochrome b_5 (b_5) involvement. Indeed, AZT reduction among six human liver samples correlated strongly with microsomal b_5 content ($r^2 = 0.96$) as well as with aggregate P450 content ($r^2 = 0.97$). Upon reconstitution, human liver b_5 plus NADH:b_5 reductase and CYP2C9 plus NADPH:P450 reductase were both effective catalysts of AZT reduction, which was also supported when CYP2A6 or CYP2E1 was substituted for CYP2C9. Kinetic analysis revealed an AZT K_m of 54 μM and V_{max} of 301 pmol/min for b_5 plus NADH:b_5 reductase and an AZT K_m of 103 μM and V_{max} of 397 pmol/min for CYP2C9 plus NADPH:P450 reductase. Our results indicate that AZT reduction to AMT by human liver microsomes involves both b_5 and P450 enzymes plus their corresponding reductases. The capacity of these proteins and b_5 to reduce AZT may be a function of their heme prothestic groups. BIOCHEM PHARMACOL **55**;6:757–766, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. AZT; AZT reduction; AMT; P450 enzymes; cytochrome b_5

AZT§ is the first nucleoside analog approved for the treatment of AIDS. AZT is converted to its triphosphate form, which then acts as a competitive inhibitor of HIV reverse transcriptase and as a chain terminator of viral DNA synthesis [1]. The most common adverse effects associated with long-term AZT therapy include hematological toxicities [2] and mitochondrial myopathy [3, 4]. The mechanisms of AZT-induced toxicity are multifactorial, and include incorporation into nuclear DNA [5, 6], inhibition of cellular DNA polymerases and exonucleases [7–9], inhibition of hemoglobin synthesis [10, 11], interference with mitochondrial respiratory chain enzyme activity [12–14], and formation of a toxic metabolite [15–17].

Besides 5′-*O*-glucuronidation, AZT also undergoes enzymatic reduction to AMT *in vivo* and *in vitro* [18, 19]. AMT is considerably more cytotoxic than AZT itself [20], which is consistent with its potent inhibition of DNA polymerase α, and subsequent inhibition of DNA synthesis [21–23]. The mechanism underlying enzymatic reduction of AZT to AMT is still unclear. Chemical reduction of AZT to AMT by thiols such as dithiothreitol and glutathione has been reported [24, 25]. The electrochemical reduction of AZT to AMT suggests involvement of a two-electron transfer mechanism [26]. Our group first suggested the involvement of P450 enzymes in AZT reduction to AMT by liver

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§ *Abbreviations:* AZT, 3′-azido-3′-deoxythymidine; AMT, 3′-amino-3′-deoxythymidine; P450, cytochrome P450; b_5, cytochrome b_5, P450 reductase, NADPH:cytochrome P450 reductase; b_5 reductase, NADH:cytochrome b_5 reductase; DLPC, L-α-dilauroylphosphatidylcholine; and KPO_4, potassium phosphate buffer.

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microsomes [18, 19]. More recently, the kinetics of AZT reduction were studied in human and rat liver microsomes, and multiple P450 enzymes were reportedly implicated [27].

The P450 enzymes constitute a superfamily of hemoproteins [28] that are particularly abundant in the hepatocyte endoplasmic reticulum. Although metabolism by these enzymes most commonly involves oxygenation of substrates, P450s also catalyze substrate reduction, particularly under conditions of low oxygen tension (i.e. anaerobiosis) [29–32]. Indeed, oxygen can inhibit P450-catalyzed reductive metabolism by competing with the enzymes for reducing equivalents [33]. Compounds containing azo, nitro, arene oxide, *N*-oxide, and alkyl halide moieties are all substrates for reduction by P450 enzymes [34]. The flavoprotein P450 reductase is also involved in these reductive drug-metabolizing reactions [35–39]. As AZT possesses an azo moiety, its reduction by P450s and/or P450 reductase is clearly possible.

In this study, we examined the human liver enzymes responsible for AZT reduction to AMT. We demonstrated that P450 enzymes and b_5 together with their corresponding reductases, namely P450 reductase and b_5 reductase, are involved in this process. Through the use of chemical inhibitors and antibodies, we also showed that P450-catalyzed AZT reduction is not attributable to any one P450 enzyme but is rather a generalized function of these hemoproteins.

MATERIALS AND METHODS

Chemicals

[5-^{3}H]AZT (14 Ci/mmol) was purchased from Moravek Biochemicals Inc. and was found to be ≥98% pure as assessed by reverse-phase HPLC (see below). Diethyldithiocarbamate was purchased from the Aldrich Chemical Co. and ketoconazole was from Janssen Life Sciences Products. Cyclosporin A was obtained from the University of Alabama at the Birmingham Hospital Pharmacy, and diazepam was a gift of Dr. R. Rahmani (INRA). Other chemicals, including unlabeled AZT, NADPH, NADH, DLPC, and the P450 substrates and inhibitors, were purchased from the Sigma Chemical Co. AtmosBags were obtained from Aldrich.

Human Liver Microsomes

Microsomes were prepared from human livers obtained from the Tissue Procurement Core Facility at the University of Alabama at Birmingham and the Liver Transplant Procurement and Distribution System (LTPADS) in Minneapolis. Protein, aggregate P450 content, and b_5 content were measured as previously described [40, 41]. Additional samples of human liver microsomes were obtained from the Keystone Skin Bank. Microsomes derived from human lymphoblast cells expressing CYP3A4 were purchased from Gentest.

Purification of Microsomal Enzymes

CYP2C9* CYP2E1, P450 reductase, and b_5 were purified from human liver microsomes as described elsewhere [41, 42]. Human liver CYP2A6 was isolated using a modification of the method described by Yun *et al.* [43]. The specific contents of CYP2C9, CYP2E1, CYP2A6, and b_5 were 7.0, 14.7, 5.8, and 41.5 nmol/mg, respectively, while the specific activity of P450 reductase was 34,550 units (10.3 nmol)/mg protein. One nanomole of P450 reductase was considered equivalent to 3370 units of activity; 1 unit was defined as that amount catalyzing NADPH-supported reduction of 1 nmol ferricytochrome *c*/min at 30° in 300 mM KPO_4 buffer (pH 7.7).

NADH:b_5 reductase was isolated from liver microsomes as follows. In brief, the b_5 reductase-enriched material recovered from tryptamine CH-Sepharose 4B with buffer C [41] was subjected to anion-exchange chromatography on DE-52. Fractions further enriched in the enzyme (as assessed by measuring NADH-supported ferricyanide reduction) were isolated by washing the DEAE-cellulose column with 100 mM Tris-acetate buffer (pH 8.0) containing 1 mM EDTA, 0.1% Triton X-100, and 30 mM NaCl. The sample was dialyzed versus 20 mM KPO_4 buffer (pH 7.25) containing 0.1% Triton X-100 to lower the ionic strength, and then was applied to a hydroxylapatite column equilibrated with 20 mM KPO_4 buffer (pH 7.25) containing 0.1% Triton X-100, and 0.1% sodium deoxycholate. The nonbinding material, which contained the bulk of the b_5 reductase activity, was concentrated 5-fold by ultrafiltration, and then applied to a 5′-ADP agarose column (N^6-linked) equilibrated with 20 mM Tris-acetate buffer, pH 7.5, containing 1 mM EDTA. The column was washed extensively with equilibration buffer, followed by 100 mM Tris-acetate buffer (pH 8.1) containing 1 mM EDTA. The bound b_5 reductase was then eluted with 50 mM Tris-acetate buffer (pH 8.1) containing 0.05% Triton X-100 and 2 mM ADP. Detergent was removed from the purified enzyme by chromatography on a small hydroxylapatite column [41]. The final b_5 reductase preparation was electrophoretically homogenous, exhibited an apparent molecular weight of 32,800, and had a specific activity of 1150 units/mg using ferricyanide as the electron acceptor. One nanomole of b_5 reductase activity was considered equivalent to 35 units of activity; 1 unit was defined as that amount catalyzing the NADH-supported reduction of 1 μmol ferricyanide/min at 22°in 100 mM KPO_4 buffer (pH 7.5).

AZT Reduction by Liver Microsomes

Initial experiments involved optimization of incubation conditions so that AZT reduction to AMT was linear with respect to incubation time and microsomal protein concentration. Subsequent metabolism studies were conducted

* The P450 enzymes described in this work are designated according to the nomenclature given in Nelson *et al.* [28].

using the following optimized reaction conditions unless noted otherwise. Incubation mixtures (0.2 mL) consisted of liver microsomes (1.4 mg protein), 0.1 mM [^{3}H]AZT, 100 mM KPO_4 buffer (pH 7.4), and 5 mM $MgCl_2$. The mixtures were prepared in glass tubes, which then were placed in the AtmosBag. The air was removed by vacuum, and the bag then filled with pure argon; the latter two steps were repeated three times to ensure anaerobiosis. After a 3-min preincubation period at 37°, reactions were initiated by adding 6 mM NADPH or NADH (with a gas-tight syringe), and were terminated after 60 min at 37° by immersing the tubes in a boiling water bath. The reaction mixtures were transferred to microfuge tubes, centrifuged at 14,000 rpm (11,000 g) for 4 min, and aliquots of the resulting supernatant (50–150 μl) were analyzed for AMT formation by the HPLC method described below. For kinetic studies with human liver microsomes, the concentration of AZT was varied from 0.01 to 10.0 mM. For chemical inhibition experiments, P450 substrates or inhibitors were added to the incubation mixture at concentrations ranging from 0.5 to 1.0 mM (except for SKF-525A, which was used at a concentration of 5 mM). For antibody inhibition studies, microsomes were preincubated with anti-human CYP2A6, anti-human CYP2E1, anti-human CYP2C9 (which cross-reacts with CYP2C8 and CYP2C19), or anti-human CYP3A4 IgG for 3 min at 37°, and then for 10 min at ambient temperature. The ratio of IgG to microsomal P450 used was 5 mg IgG/nmol P450 except in the case of anti-CYP3A4, where an antibody to P450 ratio of 4:1 was utilized. These IgG:P450 ratios had already been optimized as described elsewhere [44–48] to give maximal inhibition (≥90%) in human liver microsomes of a drug-metabolizing activity typified by the corresponding P450 enzyme; these were coumarin 7-hydroxylation with anti-CYP2A6, chlorzoxazone 6-hydroxylation with anti-CYP2E1, tolbutamide hydroxylation with anti-CYP2C9, and nifedipine pyridine metabolite formation with anti-CYP3A4. After the preincubation period, the remaining incubation components (including NADPH) were added, and the reactions were performed as described above. An equivalent amount of preimmune rabbit IgG was substituted for the immune-specific antibodies in control reactions.

AZT Reduction by Purified Hepatic Enzymes

Reconstituted enzyme systems were comprised of either: (a) 100 pmol b_5, 150 pmol b_5 reductase, and 15 μg DLPC; or (b) 100 pmol CYP2C9, 150 pmol P450 reductase, and 15 μg DLPC. In certain assays, CYP2C9 was substituted with 100 pmol CYP2E1 or CYP2A6. Other assay components included 0.1 mM [^{3}H]AZT, 6 mM NADH or NADPH, 100 mM KPO_4 buffer (pH 7.4), and 5 mM $MgCl_2$ in a final volume of 0.2 mL. Reactions were conducted as described above for liver microsomes. For kinetic studies, AZT concentrations were varied from 0.01 to 10.0 mM. Kinetic parameters (K_m and V_{max}) of AZT reduction by purified enzymes and intact liver microsomes (sample HL10 and HL14) were derived by fitting either a one- or two-component Michaelis–Menten equation to data generated from three independent experiments per specimen.

HPLC Analysis

AMT was quantitated by HPLC using a Hewlett Packard model 1090 M liquid chromatograph equipped with a diode array detector and an automatic injector. Detection of radioactivity was performed using a Flo-One β model 525A on-line radioactive flow detector (Packard Instrument). Reverse-phase chromatography was carried out on a Hypersil C_{18} column (5 μm particle size, 4.6 × 250 mm; Jones Chromatography). The mobile phase consisted of 50 mM phosphoric acid (pH 7.4), and elution was performed with a linear acetonitrile gradient changing from 0 to 30% over 35 min using a flow rate of 1 mL/min. Under these conditions, AMT and AZT exhibited retention times of 10.4 and 22.5 min, respectively. Radioactivity corresponding to AMT was converted to picomoles or nanomoles based on the specific activity of [^{3}H]AZT, and the data were expressed as picomoles or nanomoles of AMT formed per minute per milligram of microsomal protein or nanomoles of purified enzyme.

RESULTS

Reductive Metabolism of AZT to AMT by Human Liver Microsomes

A typical AZT metabolic profile obtained with human liver microsomes (sample HL10) is shown in Fig. 1. As depicted by the radiochromatogram, AMT was the sole metabolite formed under anaerobic conditions in the presence of NADPH or NADH. Aerobic conditions decreased AZT reduction over 5-fold compared with anaerobic conditions (data not shown). Similar metabolic profiles were obtained with the 21 other human liver samples tested. Rates of AMT formation (at 0.1 mM AZT and NADPH as cofactor) among these specimens ranged from 34.8 to 146.2 pmol/min/mg protein, with a mean (± SD) value of 108.5 ± 32 pmol/min/mg protein (N = 22).

Analysis of AZT reduction by human liver sample HL10 in the presence of NADPH revealed non-linear reaction kinetics (Fig. 2), suggesting the involvement of multiple microsomal enzymes. The kinetic data were best described by a two-component Michaelis–Menten model. The low K_m and V_{max} values (designated K_{m1} and V_{max1}) were 58.0 ± 10.0 μM and 107 ± 10 pmol AMT formed/min/mg protein, while the high K_m and V_{max} values (designated K_{m2} and V_{max2}) were 5.88 ± 4.31 mM and 1804 ± 670 pmol AMT formed/min/mg protein (Fig. 2). Comparable results were obtained with a second liver specimen (HL14), where K_{m1} and V_{max1} were 74 μM and 142 pmol AMT formed/min/mg protein, and K_{m2} and V_{max2} were 4.33 mM and 2607 pmol AMT formed/min/mg protein, respectively. When NADH was substituted for NADPH, specimen HL14 again exhibited biphasic AZT metabolism kinetics,

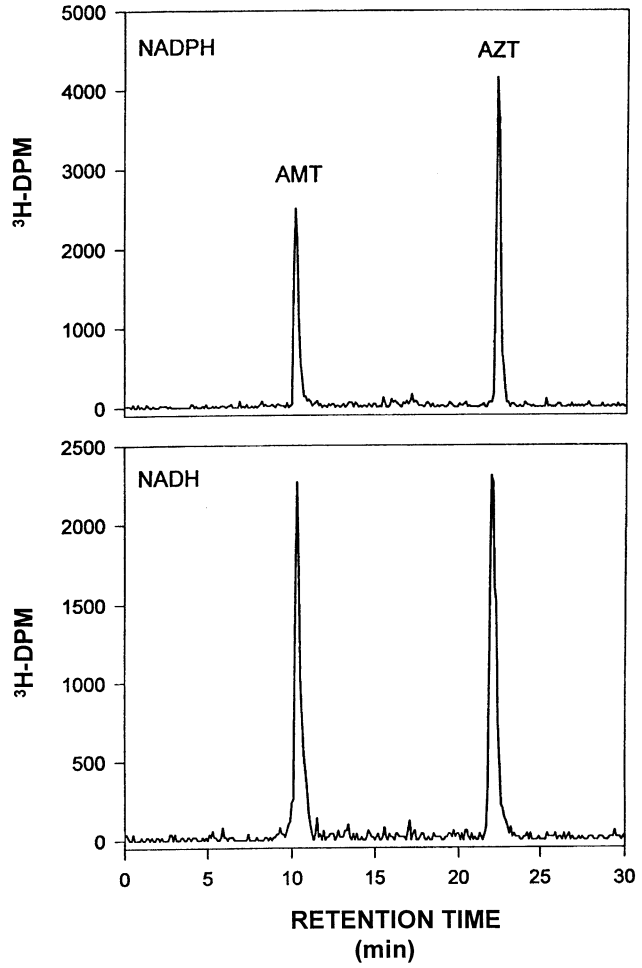

FIG. 1. HPLC analysis of AZT reduction to AMT by human liver microsomes. Liver microsomes from subject HL10 were incubated with 1 mM [^{3}H]AZT in the presence of either NADPH (upper panel) or NADH (lower panel) under anaerobic conditions. Then AMT formation assessed by HPLC with radiochromatographic detection. As shown, the metabolite AMT exhibited a retention time of 10.4 min, while the parent compound AZT exhibited a retention time of 22.5 min. Additional details of the reaction conditions are given in Materials and Methods.

with K_{m1} and V_{max1} values of 69 μM and 166 pmol AMT formed/min/mg protein, respectively, and K_{m2} and V_{max2} values of 1.84 mM and 1710 pmol AMT formed/min/mg protein, respectively.

Chemical Inhibition of Microsomal AZT Reduction

Ensuing studies were aimed at identifying the multiple enzymatic components mediating AZT reduction by human liver microsomes. Based upon previous studies [18, 19, 27], it was felt that these enzymes were either distinct P450 enzymes and/or P450 reductase. To this end, we assessed the effects of chemical agents known to be specific inhibitors and/or substrates of human P450s on microsomal AZT reductase activity (Table 1). Surprisingly, however, none of the compounds with known P450 specificity gave significant inhibition of AZT reduction. In contrast, the generalized P450 inhibitor carbon monoxide decreased AMT formation by more than 75%. Two different non-specific P450 inhibitors, namely metyrapone and ketoconazole*, also decreased rates of microsomal AZT reduction, whereas other inhibitors of this type (*n*-octylamine, SKF-525A, and cimetidine) had little effect on the reaction.

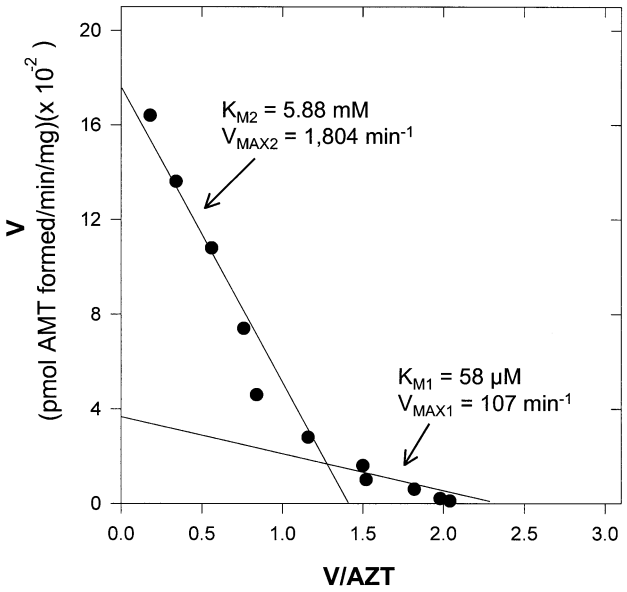

FIG. 2. Kinetic analysis of AZT reduction by human liver microsomes. AZT reduction by liver microsomes from subject HL10 was assessed as described in Materials and Methods. [^{3}H]AZT concentrations were varied from 0.01 to 10 mM, and the cofactor used as NADPH. The apparent K_m and V_{max} values shown in the figure were derived by fitting the experimental data to a two-component Michaelis–Menten equation.

Immunoinhibition of Microsomal AZT Reduction

Next, the effects of specific human P450 antibodies, namely anti-CYP2A6, anti-CYP2C9 (CYP2C8 and CYP2C19), anti-CYP2E1, and anti-CYP3A4 (CYP3A5), on AZT reduction by human liver microsomes were assessed. At an IgG:microsomal protein ratio of 5:1, none of these antibodies had an inhibitory effect on AZT conversion to AMT. At the same IgG:microsomal protein ratio, however, each antibody displayed ≥90% inhibition of a prototypic reaction catalyzed by its corresponding antigen (see Materials and Methods).

Correlation between Microsomal AZT Reduction, P450 *Content and* b_5 *Content*

The lack of inhibition of microsomal AZT reduction by chemical agents with known P450 enzyme specificity as

* Ketoconazole functions as a non-specific inhibitor of P450 enzymes at the concentration (100 μM) employed for these experiments, but is a highly specific inhibitor of CYP3A4 at lower concentrations (1–10 μM) [49, 50].

TABLE 1. Effects of P450 substrates and inhibitors on AZT reduction by human liver microsomes

Target enzyme	Substrate or Inhibitor	Concentration (mM)	% of Control*
CYP1A2	7,8-Benzoflavone	0.5	108.6
CYP2A6	Coumarin	0.5	88.7
CYP2C9/CYP2C19	Hexobarbital	0.5	91.1
	Diazepam	0.5	86.5
	Tolbutamide	0.5	87.4
CYP2D6	Quinidine	0.01	114.0
		0.5	68.3
	Debrisoquine	0.5	95.2
	Methadone	0.5	96.5
	Yohimbine	0.5	95.2
CYP2E1	Aniline	0.5	96.0
	Chlorzoxazone	1.0	80.7
	Tryptamine	2.0	71.6
	p-Nitrophenol	0.1	71.2
	Diethyldithiocarbamate	1.0	115.3
CYP3A4/CYP3A5	Erythromycin	0.5	86.5
	Troleandomycin	0.5	83.8
	Midazolam	0.5	87.3
	Cyclosporin A	0.5	129.7
Generalized CYP450	Carbon monoxide	100%	24.3
	Metyrapone	0.5	43.9
	Ketoconazole	0.1	59.2
	SKF-525A	5.0	106.8
	Cimetidine	0.5	85.6
	n-Octylamine	0.5	102.0

AZT reduction to AMT was determined in incubation mixtures (0.2 mL) containing liver microsomes (1.4 mg protein), 100 mM KPO_4 buffer (pH 7.4), 0.1 mM [^{3}H]AZT, 6 mM NADPH, and the above compounds at the indicated concentrations. For carbon monoxide, incubation mixtures were bubbled with the gas for 45 sec. Reactions were initiated with cofactor, and were terminated after 60 min at 37° under an argon atmosphere. AMT formation was then quantitated by HPLC as described under Materials and Methods. Control activity (no addition) was 106.0 pmol AMT formed/min/mg protein.

* Values denote the average of at least three separate determinations. Replicates differed from each other by 15% or less.

well as by inhibitory P450 antibodies indicated that the conversion of AZT to AMT was not mediated by distinct P450 enzymes. When viewed in light of the inhibition obtained with generalized inhibitors of P450 activity, including carbon monoxide, metyrapone, and ketoconazole, the results were more consistent with non-specific P450*-mediated reduction of AZT, possibly stemming from the hemoprotein nature of these enzymes. Furthermore, the capacity of NADH to support microsomal AZT reduction with nearly the same efficiency as NADPH suggested the involvement of a microsomal enzyme(s) other than P450. Thus, we also assessed whether reduction of AZT was related to another hemoprotein present in liver microsomes, namely b_5. As shown in Fig. 3, a strong correlation ($r^2 = 0.972$, $P < 0.001$) was found in these specimens between rates of AMT formation and aggregate P450 content (panel B). Furthermore, an excellent correlation ($r^2 = 0.960$, $P < 0.001$) was also found between rates of microsomal AMT formation and b_5 content among six human liver samples. It should be noted that NADH, the preferred electron donor substrate for b_5 reductase and b_5, was used as cofactor in these experiments.

* P450 is referred to here as in the aggregate, without implication of any one given enzyme.

AZT Reduction by Purified Liver Microsomal Enzymes

To further elucidate the respective roles of P450 and b_5 in the microsomal conversion of AZT to AMT, the capacity of these enzymes to catalyze the reduction reaction was assessed upon reconstitution with their corresponding reductases and phospholipid (DLPC). As shown in Table 2, neither b_5 reductase nor P450 reductase alone was able to reduce AZT in the presence of NADH or NADPH. Similarly, CYP2C9 (used here as a "model" P450 enzyme) and b_5 were also incapable of metabolizing AZT in the presence of only cofactor. Enzyme activity could be reconstituted only when both hemoprotein and flavoprotein were present together with cofactors. Rates of AZT reduction by the complete CYP2C9 reconstituted system were nearly identical to those exhibited by the b_5 reconstituted system. In addition, b_5 was an effective catalyst of the reaction when P450 reductase was substituted for b_5 reductase, whereas, in the case of CYP2C9, substitution of P450 reductase with b_5 reductase gave much lower rates of AZT reduction (Table 2). AZT reduction was also catalyzed by CYP2C9 or b_5 plus P450 reductase in the presence of NADH, but reduction rates were substantially less than those obtained with NADPH. Moreover, the addition of b_5 to the CYP2C9 reconstituted system resulted in a 30% stimulation of AZT reduction. In further experiments, it was found that human P450s other than CYP2C9, namely

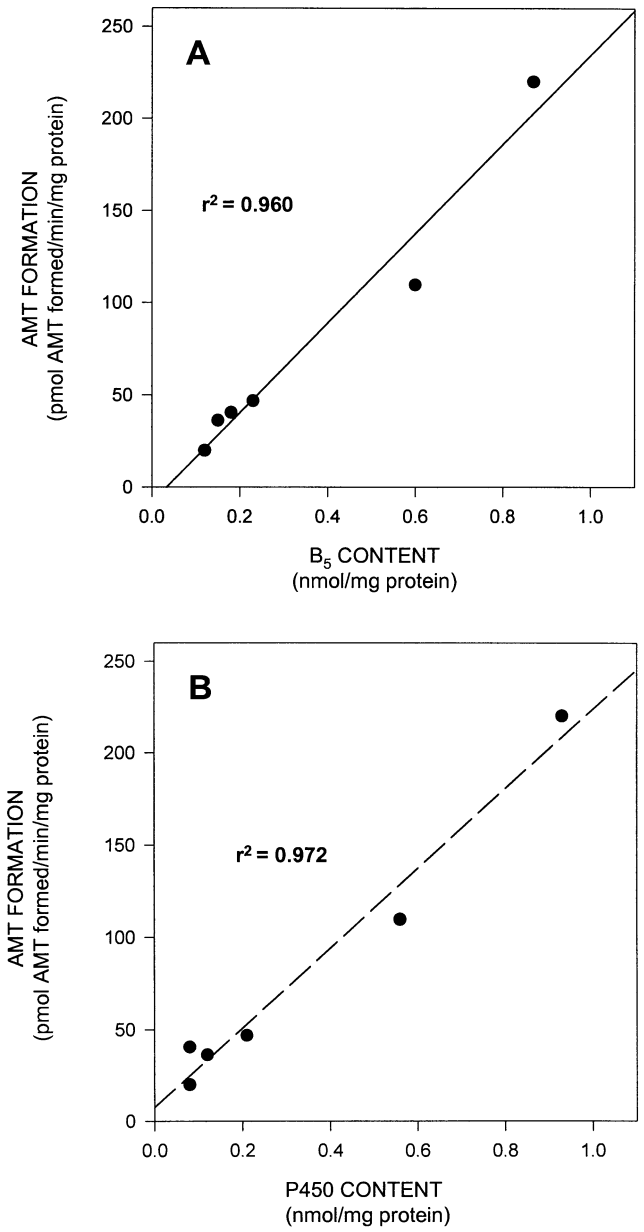

FIG. 3. Correlation between AZT reduction, b_5 content, and P450 content in human liver microsomes. AZT reduction, b_5 content, and P450 content were measured in liver microsomes from six different subjects as described under Materials and Methods. The cofactor used was NADH. Regression analysis was used to derive the correlation coefficients (r^2) shown for AZT reduction versus b_5 content (panel A) and versus P450 content (panel B).

CYP2E1 and CYP2A6, were also capable of supporting AZT reduction. CYP2E1 and CYP2A6, when reconstituted with P450 reductase and phospholipid, converted AZT to AMT at rates of 292.8 and 268.8 pmol AMT formed/min/nmol P450, respectively.

We also examined the kinetics parameters of AZT reduction for the CYP2C9 and b_5 reconstituted systems. As shown in Fig. 4, both enzymes displayed monophasic AZT reduction kinetics. The b_5 reconstituted system exhibited

TABLE 2. AZT Conversion to AMT by purified human liver CYP2C9 AND b_5

Components	AMT Formation* (pmol/min/nmol CYP2C9 or CYP2C9 b_5)
CYP2C9 + NADPH	bd†
P450 reductase +NADPH	bd
CYP2C9 + P450 reductase + NADPH	215.4 ± 31
CYP2C9 + P450 reductase + NADH	126.3 ± 43
CYP2C9 + b_5 reductase + NADH	14.0 ± 24
b_5 + NADH	bd
b_5 reductase + NADH	bd
b_5 + b_5 reductase + NADH	203.6 ± 21
b_5 + reductase + NADH‡	bd
b_5 + reductase + NADPH	bd
b_5 + P450 reductase + NADH	151.2 ± 43
b_5 + P450 reductase + NADPH	233.9 ± 49
HL-10 microsomes	168.0 ± 16, 156.8 ± 15§

AZT reduction was determined in incubation mixtures (0.2 mL) containing the following reconstituted enzyme systems: (a) 100 pmol CYP2C9, 150 pmol P450 reductase, and 15 μg DLPC; or (b) 100 pmol b_5 150 pmol b_5 reductase, and 15 μg DLPC. Other assay components included 0.1 mM [^{3}H]AZT, 6 mM NADH or NADPH, and 100 mM KPO_4 buffer (pH 7.4). Reactions were initiated with cofactor, and were terminated after 60 min at 37° under an argon atmosphere. AZT conversion to AMT was then quantitated by HPLC as described under Materials and Methods.

* Values are the means ± SD of three separate determinations.

† Below detection (< 14 pmol/min/nmol CYP2C9 or b_5).

‡ Under aerobic rather than anaerobic conditions.

§ Values are expressed as pmol AMT formed/min/nmol microsomal P450 and pmol AMT formed/min/nmol microsomal b_5, respectively.

an AZT K_m of 54 μM and a V_{max} of 301 pmol AMT formed/min/nmol b_5. The CYP2C9 reconstituted system gave similar values, with an AZT K_m of 103 μM and a V_{max} of 397 pmol AMT formed/min/nmol CYP2C9.

DISCUSSION

In this study, we examined the human liver enzymes underlying reductive metabolism of AZT, a major anti-HIV chemotherapeutic agent, to its hematotoxic derivative AMT. Studies of the kinetic parameters of this reaction in human liver microsomes revealed that a low K_m, low V_{max} and a high K_m, high V_{max} enzymatic component were involved. Due to its greater pharmacological relevance, we focused on the low K_m component, and found that two microsomal constituents, namely P450 enzymes and b_5, could catalyze reduction of AZT at low substrate concentrations. With regards to the former, however, no single P450 enzyme exhibited enhanced specificity toward AZT, as shown in both chemical and antibody-based inhibition studies. A role for b_5 in AZT reduction was indicated by the observation that NADH was as effective as NADPH in supporting the reaction in liver microsomes, and was then established in studies with purified hepatic enzymes where b_5, upon reconstitution with b_5 reductase and phospholipid, proved to be a potent catalyst of AZT reduction to AMT. CYP2C9, which was employed here as a "model"

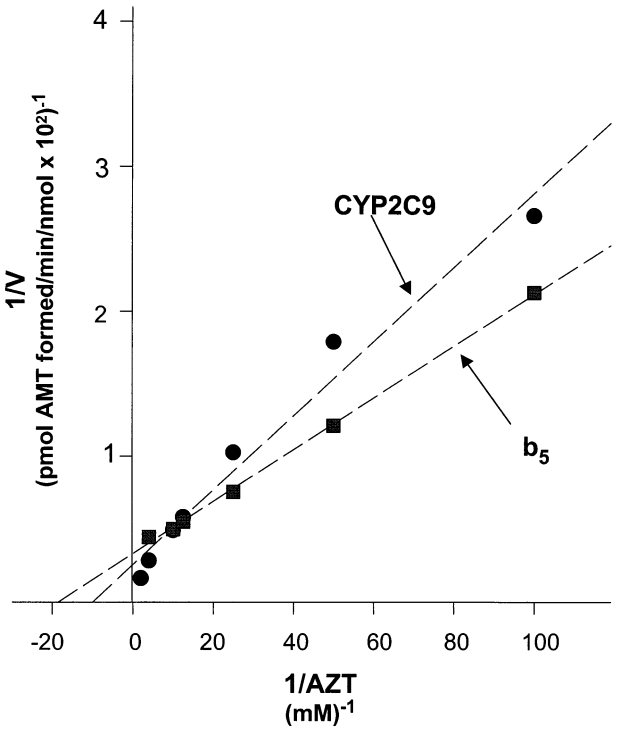

FIG. 4. Kinetic analysis of AZT reduction by purified and reconstituted microsomal enzymes. AZT reduction to AMT was assessed in incubation mixtures containing the following reconstituted systems: (a) 100 pmol b_5, 15 μg (45 μM) DLPC, and 150 pmol b_5 reductase; or (b) 100 pmol CYP2C9, 15 μg DLPC, and 150 pmol P450 reductase. Other assay components included [^{3}H]AZT (0.01 to 0.5 mM), 100 mM KPO_4 buffer (pH 7.4), and 6 mM NADPH or NADH. Reactions were initiated with cofactor, and were terminated after 60 min at 37° under an argon atmosphere. AMT formation was then quantitated by HPLC as described under Materials and Methods. Kinetic parameters (apparent K_m and V_{max}) were derived by fitting the results to a single-component Michaelis–Menten equation. Regression analysis of the lines of best fit gave r^2 = 0.974 for the CYP2C9 reconstituted system and r^2 for b_5 = 0.999.

human P450 enzyme, also proved an effective catalyst of AZT reduction upon reconstitution with P450 reductase and phospholipid, but no more so than other human P450 enzymes, including CYP2E1 or CYP2A6. Importantly, the kinetic parameters of AZT reduction derived for the b_5 and CYP2C9 reconstituted systems approximated those of the low K_m component in human liver microsomes.

A previous study by our laboratory [19] originally suggested that conversion of AZT to AMT by human liver microsomes was a P450-dependent reaction. This conclusion was based on the reaction's requirement for NADPH, the marked inhibition of AMT formation by carbon monoxide, and the relationship found between rates of microsomal AZT reduction and total P450 content [19]. Thus, our goal in the present study was to expand on these observations by defining the human P450 enzyme(s) that functioned as the primary catalyst of microsomal AZT reduction. Under anaerobic conditions, we found that human liver microsomes fortified with NADPH converted AZT to AMT with biphasic kinetics, indicating that at least two distinct enzymes participated in the reaction (Fig. 2). Such biphasic kinetics are not unique, and are exhibited by numerous therapeutic agents that undergo metabolism by more than one P450 enzyme in human liver microsomes [51]. With regard to AZT reduction, however, equivocal results were obtained in inhibition studies designed to identify the P450 enzyme(s) representing the low K_m, low V_{max} reaction component. Agents established as inhibitors or competitive substrates of specific human P450s, including CYP1A2, CYP2A6, CYP2C9/CYP2C19, CYP2D6, CYP2E1, and CYP3A4 [49], failed to give inhibition, reflecting participation of a specific P450 enzyme in microsomal AZT reduction (Table 1). Moreover, P450 antibodies known to markedly inhibit reactions catalyzed by their corresponding antigens [44–48] were also without effect on AZT reduction. Yet, significant inhibition was obtained with several of the generalized P450 inhibitors, including carbon monoxide (76%), metyrapone (56%), and ketoconazole (41%) (Table 1), suggesting that AZT is reduced by P450 enzymes in a non-specific manner. Similar results were obtained by Eagling *et al.* [27], who, in their study of AZT reduction by human liver microsomes, also found that carbon monoxide and ketoconazole were strong inhibitors of the reaction, whereas CYP1A2, CYP2C9, CYP2E1, and CYP3A4 substrates were ineffective. That P450 enzymes were actually involved in AZT reduction, however, was indicated by the excellent correlation (r^2 = 0.972) observed between rates of AMT formation and aggregate P450 content in human liver microsomes (Fig. 3) and by the capacity of purified human P450s to catalyze this reaction, as discussed below.

The involvement of b_5 in microsomal AZT reduction was first suggested in experiments showing that rates of AZT reduction in the presence of NADH were rather similar to those obtained with NADPH (see Results). NADH is the native electron donor for b_5 via the transfer of reducing equivalent through b_5 reductase and, to a lesser extent, P450 reductase [52]. Another indication for b_5 involvement was provided by the incomplete inhibition of NADPH-mediated AZT reduction by carbon monoxide, despite the fact that the 45-sec gassing period used is sufficient to form ferric carbonyl complexes with at least 90% of the total P450 present in human liver microsomes [53]; b_5 fails to form such complexes with carbon monoxide, and the transfer reducing equivalents derived from NADPH to b_5; via P450 reductase [52] would also remain unaffected by carbon monoxide. The excellent correlation (r^2 = 0.960) observed between rates of microsomal AZT reduction and b_5 content (Fig. 3) furnished yet another indication of the participation of this hemoprotein in the conversion of AZT to AMT. However, the most direct evidence for b_5 involvement in AZT reduction was provided in experiments utilizing purified enzymes, where it was shown that b_5 reconstituted with b_5 reductase and phospholipid reduced AZT to AMT at high rates (Table 2). AZT reduction by the b_5/b_5 reductase system showed an

absolute dependence upon NADH, although when b_5 was reconstituted with P450 reductase, both NADH and NADPH could serve as cofactors and the system was as active toward AZT as when reconstituted with b_5 reductase. Studies with purified enzymes also revealed that the "model" human P450 enzyme CYP2C9, upon reconstitution with P450 reductase and phospholipid, was likewise an efficient catalyst of AZT reduction (Table 2), metabolizing this substrate at rates similar to the b_5/b_5 reductase system. Contrasting with the latter reconstituted system, however, the CYP2C9/P450 reductase system metabolized AZT in the presence of either NADPH or NADH, and was not nearly as active upon the substitution of reductases. CYP2C9 was not the only human P450 capable of AZT reduction, as even higher rates (25–35%) of AMT formation were supported by CYP2E1 and CYP2A6 reconstituted systems (see Results). Although purified human CYP3A4 was not available to us, the ability of this abundant hepatic P450 enzyme to reduce AZT at rates comparable to those obtained with the reconstituted systems was confirmed using microsomes derived from human lymphoblasts expressing CYP3A4 (data not shown). That AZT reduction by human b_5 and P450s was an activity inherent to these enzymes and not to hemoproteins in general was indicated by the failure of both hemoglobin and cytochrome *c* to promote AMT formation (data not shown).

Due to the ineffectiveness of both chemical and antibody-based inhibitors, we attempted to employ kinetic analysis for assessing the respective roles of b_5 and P450 enzymes in microsomal AZT reduction. The b_5/b_5 reductase and CYP2C9/P450 reductase reconstituted systems both exhibited monophasic Lineweaver–Burk plots (Fig. 4), with similar Michaelis constants (54 vs 103 μM) and V_{max} values (301 vs 397 pmol AMT formed/min/nmol enzyme) (Fig. 4). Importantly, the kinetic parameters of AZT reduction displayed by the b_5/b_5 reductase and CYP2C9/P450 reductase reconstituted systems resembled those of the low K_m component found in human liver microsomes. However, their close similarity to each other obviated any conclusion as to which hemoprotein is the predominant AZT reductase at low substrate concentrations. Assuming that: (a) the characteristics of AZT reduction by CYP2C9 resemble those of other human P450 enzymes; and (b) the kinetics exhibited by purified b_5 and CYP2C9 are indeed reflective of their function in intact microsomes, then the relative capacities of b_5 and P450 enzymes to reduce AZT may be directly related to their concentration in hepatic endoplasmic reticulum. Moreover, it should be emphasized that the AZT K_m values determined here for b_5, CYP2C9, and human liver microsomes appear relevant to the *in vivo* disposition of the drug, as plasma concentrations of AZT reach 10 μM after its oral administration [15, 17].

Based upon the results presented herein, we have proposed a mechanism for the anaerobic reduction of AZT to AMT in human liver microsomes (Fig. 5). In this scheme, P450 enzymes and/or b_5 convert the azido moiety of AZT to an amino group utilizing reducing equivalents derived from

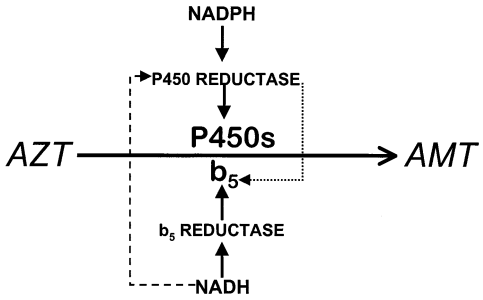

FIG. 5. Proposed mechanism of AZT reduction to AMT by human liver microsomes. The major and minor pathways of AZT reduction by P450 enzymes and b_5 are represented by solid and broken lines, respectively.

NADPH and/or NADH, with either P450 reductase or b_5 reductase serving as the electron transfer flavoprotein. Oxygen is a strong inhibitor of AZT reduction (Table 2), although the reaction still proceeds in its presence [27]. Our proposed mechanism is in accordance with the flow of electrons that reportedly occurs during P450-mediated (and b_5 enhanced) monooxygenase reactions [50]. Only the enzymes functioning at low (≤0.1 mM) AZT concentrations are shown, as those underlying the high K_m, high V_{max} component in human liver microsomes were not defined in this study.

In summary, the reductive metabolism of AZT to AMT by human liver microsomes appears to be a non-specific enzymatic process mediated by both P450 enzymes and b_5 together with their corresponding reductases. No particular P450 enzyme appears to play a prevailing role in microsomal AZT reduction, and the capacity of these proteins and b_5 to reduce AZT may be related to their unique heme prosthetic groups. Moreover, this study is the first to demonstrate that under anaerobic conditions, b_5 is directly involved in the reductive metabolism of xenobiotics. Although only a minor metabolite of AZT, AMT is of toxicological importance since it is markedly more injurious to human hematopoietic progenitor cells than AZT [20]. As the major adverse effect of AZT in patients is hematological toxicity, resulting in anemia and granulocytopenia, such toxicity may stem, at least in part, from P450- and b_5-catalyzed AMT formation in liver or in bone marrow cells themselves.

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