Biochemical Pharmacology, Vol. 52, pp. 301–309, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00208-2

Requirements for Cytochrome b_5 in the Oxidation of 7-Ethoxycoumarin, Chlorzoxazone, Aniline, and *N*-Nitrosodimethylamine by Recombinant Cytochrome P450 2E1 and by Human Liver Microsomes

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ABSTRACT. NADH-dependent 7-ethoxycoumarin O-deethylation activities could be reconstituted in systems containing cytochrome b_5 (b_5), NADH-b_5 reductase, and bacterial recombinant P450 2E1 in 100 mM potassium phosphate buffer (pH 7.4) containing a synthetic phospholipid mixture and cholate. Replacement of NADH-b_5 reductase with NADPH-P450 reductase yielded a 4-fold increase in 7-ethoxycoumarin O-deethylation activity, and further stimulation (~1.5-fold) could be obtained when NADPH was used as an electron donor. Removal of b_5 from the NADH- and NADPH-supported systems caused a 90% loss of 7-ethoxycoumarin O-deethylation activities in the presence of NADPH-P450 reductase, but resulted in complete loss of the activities in the absence of NADPH-P450 reductase. K_m values were increased and V_{max} values were decreased for 7-ethoxycoumarin O-deethylation when b_5 was omitted from the NADPH-supported P450 2E1-reconstituted systems. Requirements for b_5 in P450 2E1 systems were also observed in chlorzoxazone 6-hydroxylation, aniline *p*-hydroxylation, and *N*-nitrosodimethylamine *N*-demethylation. In human liver microsomes, NADH-dependent 7-ethoxycoumarin O-deethylation, chlorzoxazone 6-hydroxylation, aniline *p*-hydroxylation, and *N*-nitrosodimethylamine *N*-demethylation activities were found to be about 55, 41, 33, and 50%, respectively, of those catalyzed by NADPH-supported systems. Anti-rat NADPH-P450 reductase immunoglobulin G inhibited 7-ethoxycoumarin O-deethylation activity catalyzed by human liver microsomes more strongly in NADPH- than NADH-supported reactions, while anti-human b_5 immunoglobulin G inhibited microsomal activities in both NADH- and NADPH-supported systems to similar extents. These results suggest that b_5 is an essential component in P450 2E1-catalyzed oxidations of several substrates used, that about 10% of the activities occur via P450 2E1 reduction by NADPH-P450 reductase in the absence of b_5, and that the NADH-supported system contributes, in part, to some reactions catalyzed by P450 2E1 in human liver microsomes. BIOCHEM PHARMACOL 52;2: 301–309, 1996.

KEY WORDS. P450 2E1; cytochrome b_5; 7-ethoxycoumarin; chlorzoxazone; aniline; *N*-nitrosodimethylamine; electron transfer; reconstitution

P450 2E1 is one of the major P450 enzymes in human liver microsomes that catalyze oxidation of a variety of xenobiotic chemicals including drugs, toxic chemicals, and carcinogens [1, 2]. In experimental animal models, P450 2E1 has been shown to be induced by several chemicals including ethanol, acetone, and isoniazid and by chemically induced diabetes [3–5]. Purified P450 2E1 enzymes isolated from liver microsomes of animals treated with these inducers have been reported to have typical catalytic activities towards a variety of low-molecular-weight chemicals and to show requirements of b_5 for maximal catalytic activities [6–8]. Human P450 2E1 has also been purified from liver microsomes and characterized; the substrate specificities of human P450 2E1 thus isolated have been reported to be very similar in catalytic functions to those of animal orthologs [9, 10].

Some of the drug-oxidation reactions catalyzed by reconstituted monooxygenase systems containing purified P450 enzymes have been shown to require particular reconstitution conditions in order to exhibit high catalytic activities [11, 12]. For example, Gillam *et al.* [13, 14] and Ueng *et al.* [15] have demonstrated recently that a system containing a phospholipid mixture (three species of synthetic phospho-

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Received 27 November 1995; accepted 30 January 1996.

lipids), cholate, GSH, $MgCl_2$, b_5,[‖] and NADPH-P450 reductase is effective in improving some of the P450 3A4-dependent drug-oxidation reactions such as testosterone 6β-hydroxylation, nifedipine oxidation, and aflatoxin B_1 3α-hydroxylation and 8,9-epoxidation. Mechanisms underlying stimulation of catalytic activities of P450 3A-related reactions by these components have not been studied fully; however, we recently obtained evidence suggesting that an acceleration of first electron flow from NADPH-P450 reductase to P450 3A4-substrate complex due to interaction with b_5 is one of the mechanisms for the stimulation of testosterone 6β-hydroxylation and nifedipine oxidation by b_5 in reconstituted systems [16, 17].

As mentioned above, b_5 has also been reported to function as a stimulatory factor in drug-oxidation reactions catalyzed by P450 2E1 in reconstituted systems [7, 18]. However, there have been no systematic studies on the roles of several components including phospholipid mixtures, cholate, GSH, and $MgCl_2$ as well as microsomal electron transfer components such as b_5, NADH-b_5 reductase, and NADPH-P450 reductase in the oxidation of a variety of chemicals catalyzed by P450 2E1 in reconstituted systems.

We studied the effects of NADH and NADPH on the oxidations of 7-ethoxycoumarin O-deethylation by reconstituted human P450 2E1 systems containing rabbit NADH-b_5 reductase, rat or rabbit NADPH-P450 reductase, and rabbit or human b_5. We also examined the effects of antibodies raised against NADPH-P450 reductase and b_5 in the oxidation activities catalyzed by human liver microsomes in order to explore the roles of these components in the reactions. The effects of b_5 on the oxidations of other substrates such as chlorzoxazone, aniline, and *N*-nitrosodimethylamine by the reconstituted P450 2E1 system and by human liver microsomes are also reported.

MATERIALS AND METHODS

Chemicals

7-Ethoxycoumarin was obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and 7-hydroxycoumarin was from the Katayama Chemical Co., Osaka, Japan. Chlorzoxazone and 6-hydroxychlorzoxazone were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Nitrosodimethylamine, aniline, and *p*-aminophenol were from the Wako Pure Chemical Co., Osaka, Japan. Other chemicals used were from the same sources as described previously or of the highest quality commercially available [19].

Enzymes

Liver microsomes from human sample HL-11, which contains high levels of P450 2E1 [19], were prepared in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% glycerol (v/v) as described previously [20]. Recombinant P450s 2E1, 1A1, and 1A2 were purified from membranes of *Escherichia coli* into which P450s 2E1, 1A1, and 1A2 cDNA had been introduced [21–23]. Rat P450s 1A1, 1A2, and 2B1 were purified to homogeneity from liver microsomes as described previously [24]. Rabbit NADH-b_5 reductase, rabbit and rat NADPH-P450 reductases, and rabbit and human b_5 were purified from liver microsomes of phenobarbital-treated rabbits or of human liver microsomes by the methods of Mihara and Sato [25], Yasukochi and Masters [26], and Shimada *et al.* [27], respectively.

Antibodies

Rabbit anti-human b_5 and anti-rat NADPH-P450 reductase antibodies were prepared, and the IgG fractions were obtained as described [28]. Anti-human b_5 IgG was purified further with a b_5-conjugated affinity column according to the methods of Noshiro and Omura [29] as described previously [17].

Enzyme Assays

Standard incubation mixtures (final volume of 0.25 mL) for 7-ethoxycoumarin O-deethylation by reconstituted P450 2E1 systems consisted of 0.040 μM P450 2E1, 0.080 μM NADPH-P450 reductase, and 0.080 μM b_5 in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM cholate, 20 μg of a phospholipid mixture (L-α-dilauroyl-sn-glycero-3-phosphocholine, L-α-dioleoyl-sn-glycero-3-phosphocholine, and L-α-phosphatidyl-L-serine, 1:1:1, by wt) per mL, 1.0 mM NADH and/or NADPH or an NADPH-generating system [24], and 200 μM 7-ethoxycoumarin. When required, 0.080 μM NADH-b_5 reductase was included in the reaction mixture. Liver microsomal incubations included microsomes (0.10 mg protein/mL) of human sample HL-11 (in place of reconstituted components) in 100 mM potassium phosphate buffer (pH 7.4) containing 1.0 mM NADH and/or NADPH and 7-ethoxycoumarin. We have reported previously that 7-ethoxycoumarin O-deethylation is catalyzed by both P450 1A2 and P450 2E1 in human liver microsomes [30]. Since P450 2E1 has been identified to be a high K_m enzyme for the O-deethylation reaction, we determined the activities using a substrate (7-ethoxycoumarin) concentration of 200 μM in this study. Reactions were started by the addition of NADH and/or NADPH, incubated at 37° for 15 min, and terminated by adding 25 μL of aqueous 10% trichloroacetic acid (w/v). The metabolites formed were extracted with 1.5 mL of methylene chloride. The mixtures were centrifuged at 3000 *g* for 5 min, and aliquots (usually 1.0 mL) of the organic layer (lower layer) were re-extracted with 3.0 mL of 30 mM sodium borate (pH 9.0). The formation of 7-hydroxycoumarin was determined fluorometrically with a Shimadzu RF-5000 spectrophotometer (Shimadzu, Kyoto, Japan).

Rates of chlorzoxazone 6-hydroxylation by reconstituted systems and by human liver microsomes were determined

[‖]*Abbreviations*: b_5, cytochrome b_5; DLPC, L-α-dilauroyl-*sn*-glycero-3-phosphocholine; IgG, immunoglobulin G; and GSH, (reduced) glutathione.

by a reverse-phase HPLC according to methods described previously [31, 32]. Incubation conditions for chlorzoxazone 6-hydroxylation were the same as those for 7-ethoxycoumarin O-deethylation except that the substrate concentration used was 500 μM. Aniline *p*-hydroxylation and *N*-nitrosodimethylamine *N*-demethylation activities were determined by incubating 1 mM aniline and *N*-nitrosodimethylamine, respectively, with 5 times the concentration of the P450 2E1-reconstituted systems used for oxidations of 7-ethoxycoumarin or chlorzoxazone or with human liver microsomes (1 mg protein/mL). Incubations were conducted for 15 and 30 min, respectively, where the product formation was increased linearly with time as determined by methods described previously [20, 33].

Other Assays

P450 and b_5 were estimated spectrally by the method of Omura and Sato [34]. Protein concentrations were estimated by the method of Lowry *et al.* [35].

Kinetic parameters for the hydroxylation reactions by P450 2E1 were estimated using a computer program (KaleidaGraph, Synergy Software, Reading, PA, U.S.A.) designed for nonlinear regression analysis of a hyperbolic Michaelis–Menten equation.

RESULTS

Reconstitution of 7-Ethoxycoumarin O-Deethylation in NADH- and NADPH-Supported P450 2E1 Systems

7-Ethoxycoumarin O-deethylation activities were reconstituted in systems containing recombinant P450 2E1, NADPH-P450 reductase, and b_5 in potassium phosphate or HEPES buffer containing a lipid mixture and cholate and NADH or NADPH as electron donors (Fig. 1). Product formation increased linearly with increased incubation time in both NADH and NADPH systems and was dependent on the concentrations of P450 2E1 up to 40 nM in the NADPH system; the activities obtained in the NADH-supported system were found to be about 55% of those obtained in NADPH-supported systems (Fig.1, A and B). Potassium phosphate buffer was more effective than the potassium HEPES buffer in the NADPH-supported P450 2E1 system (Fig. 1C). Maximal activities were obtained when the molar ratio of b_5 or NADPH-P450 reductase to P450 2E1 was increased to more than 2 (Fig. 1, D and E).

Requirements for several components of 7-ethoxycoumarin O-deethylation by reconstituted P450 2E1 systems were determined (Table 1). A lipid mixture was more suitable than DLPC, and b_5 was a critical component for reconstituting O-deethylation activities in both systems containing potassium phosphate and HEPES buffers. $MgCl_2$ and GSH could serve as stimulatory factors in the reconstituted O-deethylation activities only in the potassium HEPES buffer. Since neither $MgCl_2$ nor GSH was required for 7-ethoxycoumarin O-deethylation in reconstituted P450 2E1 systems containing potassium phosphate buffer, we did not add these two components and used 100 mM potassium phosphate buffer rather than the HEPES buffer in the following standard reconstituted systems.

Roles of NADH-b_5 reductase, NADPH-P450 reductase, and b_5 in 7-ethoxycoumarin O-deethylation were determined under optimal conditions in NADH- and NADPH-

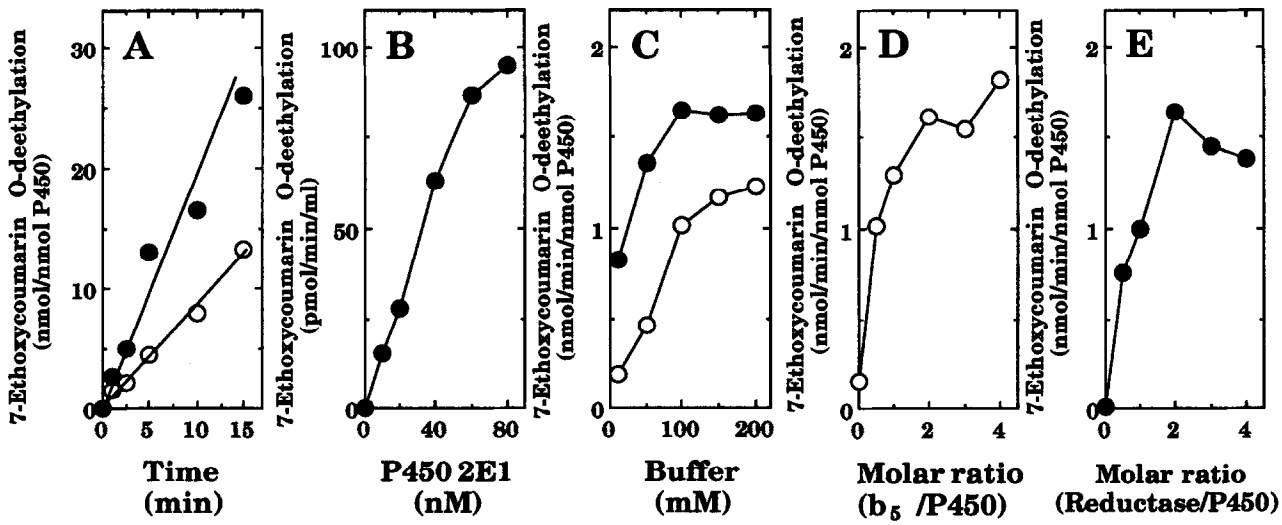

FIG. 1. Effects of incubation time on NADH-dependent (○) and NADPH-dependent (●) 7-ethoxycoumarin O-deethylation (A) and of concentrations of P450 2E1 (B), potassium phosphate (●) or HEPES (○) buffer (pH 7.4) (C), and molar ratio of b_5 to P450 2E1 (D) and NADPH-P450 reductase to P450 2E1 (E) on NADPH-dependent 7-ethoxycoumarin O-deethylation in reconstituted monooxygenase systems containing P450 2E1. A standard incubation mixture consisted of 40 nM P450 2E1, 80 nM NADPH-P450 reductase, 80 nM b_5, and 100 mM phosphate buffer containing 1.0 mM NADH or NADPH (A) and an NADPH-generating system (B, C, D, and E) and other reconstituted components as described in Materials and Methods. In panel D, 2 molar excess of NADPH-P450 reductase to P450 2E1 was included, and in panel E, 2 molar excess of b_5 to P450 2E1 was present in the reaction mixture. The incubation time used was 15 min in all cases except for panel A. Data are means of duplicate determinations.

TABLE 1. Effects of various components on reconstituted 7-ethoxycoumarin O-deethylation in systems containing recombinant P450 2E1 and NADPH-P450 reductase in the presence of an NADPH-generating system

System					7-Ethoxycoumarin O-deethylation
Lipid	Buffer	b_5	$MgCl_2$	GSH	(nmol/min/nmol P450)
Mix	HEPES	+	+	+	1.69 ± 0.25 (100)
DLPC	HEPES	+	+	+	0.85 ± 0.09 (50)
Mix	HEPES	−	+	+	0.13 ± 0.02 (8)
Mix	HEPES	+	−	+	0.99 ± 0.14 (61)
Mix	HEPES	+	+	−	1.24 ± 0.19 (73)
Mix	KP_i	+	+	+	1.56 ± 0.18 (100)
Mix	KP_i	−	−	−	0.15 ± 0.02 (10)
Mix	KP_i	+	−	+	1.55 ± 0.20 (99)
Mix	KP_i	+	−	−	1.65 ± 0.24 (106)
DLPC	KP_i	+	−	−	0.54 ± 0.08 (35)

7-Ethoxycoumarin O-deethylation activities were determined in the reaction mixture (0.25 mL) containing P450 2E1 (40 nM), NADPH-P450 reductase (80 nM), b_5 (80 nM), lipid mixture or DLPC (20 μg/mL), sodium cholate (0.50 mM), GSH (3 mM), $MgCl_2$ (30 mM), and 7-ethoxycoumarin (0.2 mM) in 100 mM potassium phosphate (KP_i) or HEPES buffer (pH 7.4) containing an NADPH-generating system. Data represent means ± range of duplicate determinations. Numbers in parentheses indicate percent of the maximal activities.

supported P450 2E1 systems (Table 2). NADH-dependent 7-ethoxycoumarin O-deethylation activities could be reconstituted in systems containing b_5, NADH-b_5 reductase, and P450 2E1. Both b_5 and NADH-b_5 reductase were required for 7-ethoxycoumarin O-deethylation activity. Replacement of NADH-b_5 reductase with NADPH-P450 reductase yielded a 4-fold increase in the 7-ethoxycoumarin O-deethylation activity, and further stimulation (~1.5-fold) could be obtained when NADPH was used as an electron donor instead of NADH. Removal of b_5 from the NADH- and NADPH-supported systems caused a 90% loss of 7-ethoxycoumarin O-deethylation activities in the presence of NADPH-P450 reductase, but resulted in a complete loss of the activity in the absence of NADPH-P450 reductase.

TABLE 2. Effects of NADH-b_5 reductase, NADPH-P450 reductase, and b_5 on NADH- and NADPH-dependent 7-ethoxycoumarin O-deethylation in reconstituted systems containing recombinant P450 2E1

System				7-Ethoxy-coumarin O-deethylation
Cofactor	NADH-b_5 reductase	NADPH-P450 reductase	b_5	(nmol/min/nmol P450)
NADH	+	−	+	0.27 ± 0.04
NADH	−	−	+	<0.01
NADH	+	−	−	<0.01
NADH	−	+	+	1.03 ± 0.14
NADH	−	+	−	0.09 ± 0.02
NADH	+	+	+	1.05 ± 0.14
NADH	+	+	−	0.15 ± 0.02
NADPH	+	−	+	0.26 ± 0.03
NADPH	−	−	+	<0.01
NADPH	+	−	−	<0.01
NADPH	−	+	+	1.62 ± 0.21
NADPH	−	+	−	0.15 ± 0.03
NADPH	+	+	+	1.59 ± 0.24
NADPH	+	+	−	0.16 ± 0.05

7-Ethoxycoumarin O-deethylation activities were determined in the reaction mixture (0.25 mL) containing P450 2E1 (40 nM), NADH-b_5-reductase, and/or NADPH-P450 reductase (80 nM), b_5 (80 nM), lipid mixture (20 μg/mL), sodium cholate (0.50 mM), and 7-ethoxycoumarin (0.2 mM) in 100 mM potassium phosphate buffer (pH 7.4) containing NADH or NADPH (1.0 mM). Data represent means ± range of duplicate determinations.

Kinetic Analysis of 7-Ethoxycoumarin O-Deethylation by Reconstituted P450 2E1 Systems in the Presence or Absence of b_5

Effects of b_5 on the kinetics of 7-ethoxycoumarin O-deethylation were examined in NADPH-supported P450 2E1 systems (Fig. 2). In the absence of b_5, the K_m and V_{max} (k_{cat}) values were determined to be 0.39 mM and 0.37 nmol product formed/min/nmol P450 in reconstituted system. Addition of b_5 to the system caused a reduction in the K_m value (0.11 mM) and an increase in the V_{max} value (1.9 nmol product formed/min/nmol P450), thus causing a marked increase in the V_{max}/K_m ratio from 0.95 to 17.3.

Effects of b_5 on the Reconstituted 7-Ethoxycoumarin O-Deethylation by Recombinant Human P450 1A1, 1A2, and 2E1 and Purified Rat P450 1A1, 1A2, and 2B1

The effects of b_5 on 7-ethoxycoumarin O-deethylation by reconstituted monooxygenase systems containing several

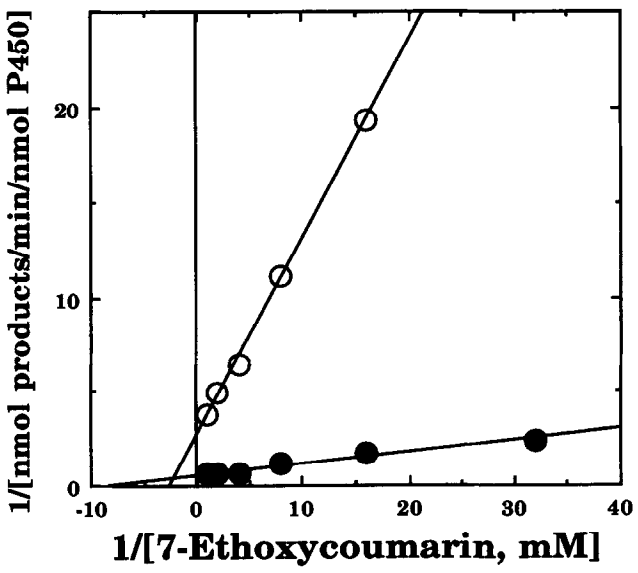

FIG. 2. Lineweaver-Burk plots of 7-ethoxycoumarin O-deethylation by reconstituted systems containing P450 2E1 and NADPH-P450 reductase in the absence (○) or presence (●) of b_5. A standard incubation mixture with various concentrations of 7-ethoxycoumarin was used. The values presented are means of duplicate determinations at each substrate concentration.

recombinant human P450 enzymes including P450 1A1, 1A2, and 2E1 and purified P450 1A1, 1A2, and 2B1 enzymes isolated from liver microsomes of rats treated with phenobarbital and 3-methylcholanthrene were examined in NADH- and NADPH-supported systems (Table 3). In all cases except for the system containing human P450 2E1, NADH was a very poor electron donor for supporting 7-ethoxycoumarin O-deethylation. We also found that the stimulatory effects of b_5 were detected only in the system containing P450 2E1, in NADPH-supported reconstituted systems.

Roles of b_5 in the 7-Ethoxycoumarin O-Deethylation by Human Liver Microsomes

Liver microsomes from human sample HL-11 were used for studies on the role of b_5 in the 7-ethoxycoumarin O-deethylation activities, since this microsomal preparation was the highest in levels of P450 2E1 in liver microsomes of 60 human samples examined [19]. NADH served as an effective electron donor for 7-ethoxycoumarin O-deethylation by human liver microsomes; the activities by the NADH system were about 60% of those by the NADPH system (data not shown). There were no marked increases in the 7-ethoxycoumarin O-deethylation activities when NADH was added to the NADPH-supported systems.

The effects of antibodies raised against human b_5 and NADPH-P450 reductase on the 7-ethoxycoumarin O-deethylation by human liver microsomes were studied (Fig. 3). Anti-NADPH-P450 reductase IgG was more effective in inhibiting 7-ethoxycoumarin O-deethylation catalyzed by liver microsomes in the system containing NADPH than that with NADH. The inhibitory effects of anti-b_5 IgG were essentially the same in both NADH- and NADPH-supported systems.

TABLE 3. Effects of b_5 on reconstituted 7-ethoxycoumarin O-deethylation in systems containing different forms of human and rat P450 enzymes and NADPH-P450 reductase

P450	System: Cofactor	System: b_5	7-Ethoxycoumarin O-deethylation (nmol/min/nmol P450)	(%)
Human				
P450 1A1	NADPH	+	4.00 ± 0.33	(100)
	NADPH	−	4.49 ± 0.58	(112)
	NADH	+	0.33 ± 0.05	(8)
P450 1A2	NADPH	+	0.12 ± 0.02	(100)
	NADPH	−	0.11 ± 0.01	(92)
	NADH	+	<0.01	(<1)
P450 2E1	NADPH	+	1.57 ± 0.24	(100)
	NADPH	−	0.15 ± 0.02	(10)
	NADH	+	0.89 ± 0.10	(57)
Rat				
P450 1A1	NADPH	+	3.17 ± 0.46	(100)
	NADPH	−	3.84 ± 0.50	(121)
	NADH	+	0.30 ± 0.05	(9)
P450 1A2	NADPH	+	0.15 ± 0.02	(100)
	NADPH	−	0.16 ± 0.03	(107)
	NADH	+	<0.01	(<1)
P450 2B1	NADPH	+	1.03 ± 0.12	(100)
	NADPH	−	1.02 ± 0.11	(99)
	NADH	+	<0.01	(<1)

7-Ethoxycoumarin O-deethylation activities were determined in the reaction mixture (0.25 mL) containing P450 (40 nM), NADPH-P450 reductase (80 nM), b_5 (80 nM), lipid mixture (20 μg/mL), sodium cholate (0.50 mM), and 7-ethoxycoumarin (0.2 mM) in 100 mM potassium phosphate buffer (pH 7.4) containing NADH or NADPH (1.0 mM). Data represent means ± range of duplicate determinations. Numbers in parentheses indicate percent of the activities.

Effects of b_5 on Oxidations of Chlorzoxazone, Aniline, and N-Nitrosodimethylamine by Reconstituted P450 2E1 Systems and by Human Liver Microsomes

Three substrates—chlorzoxazone, aniline, and *N*-nitrosodimethylamine—which are reported to be catalyzed mainly by P450 2E1 in liver microsomes of experimental animals and humans [3, 5, 19, 31, 36, 37]—were selected for further analysis of the effects of b_5 in the catalytic activities by the reconstituted P450 2E1 system and by human liver microsomes. With the three substrates examined, NADH could serve as an effective electron donor for catalytic activities; the chlorzoxazone 6-hydroxylation, aniline *p*-hydroxylation, and *N*-nitrosodimethylamine *N*-demethylation activities in NADH-supported systems were found to be about 38, 74, and 86%, respectively, of those catalyzed by NADPH-supported systems in the presence of b_5 (Table 4). As in the case of 7-ethoxycoumarin O-deethylation by reconstituted P450 2E1 system, b_5 was also a critical component for oxidations of chlorzoxazone, aniline, and *N*-nitrosodimethylamine.

The effects of NADH and/or NADPH on chlorzoxazone 6-hydroxylation, aniline *p*-hydroxylation, and *N*-nitrosodimethylamine *N*-demethylation activities catalyzed by human liver microsomes were determined (Table 5). Activities for chlorzoxazone 6-hydroxylation, aniline *p*-hy-

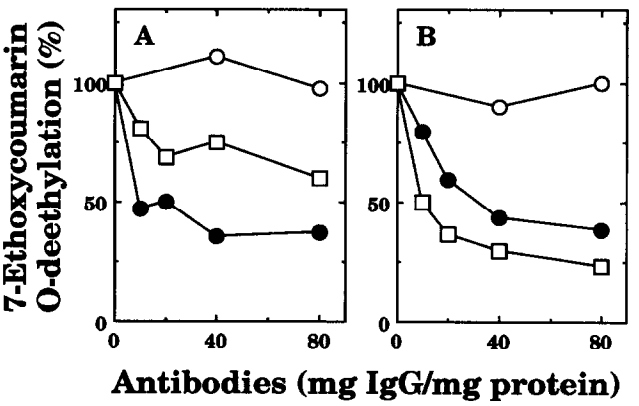

FIG. 3. Effects of preimmune IgG (○), anti-b_5 IgG (●), and anti-NADPH-P450 reductase IgG (□) on NADH-dependent (A) and NADPH-dependent (B) 7-ethoxycoumarin O-deethylation activities catalyzed by liver microsomes of human sample HL-11. Control activities in the absence of antibodies were 0.55 and 1.02 nmol product formed/min/nmol P450 for 7-ethoxycoumarin O-deethylation with NADH and NADPH, respectively. Data are means of duplicate determinations.

TABLE 4. Effects of b_5 on reconstituted monooxygenase activities in systems containing recombinant P450 2E1 and NADPH-P450 reductase

System		Chlorzoxazone 6-hydroxylation	Aniline p-hydroxylation (nmol product formed/min/nmol P450)	N-Nitrosodimethylamine N-demethylation
Cofactor	b_5			
NADPH	+	5.78 ± 0.33 (100)	11.2 ± 0.92 (100)	4.00 ± 0.40 (100)
NADPH	-	0.41 ± 0.05 (7)	4.15 ± 0.55 (37)	0.36 ± 0.06 (9)
NADH	+	2.18 ± 0.33 (38)	8.25 ± 0.99 (74)	3.43 ± 0.45 (86)

Drug monooxygenase activities were determined in the reaction mixture (0.25 mL) containing P450 2E1 (40 nM for chlorzoxazone 6-hydroxylation, and 200 nM for aniline p-hydroxylation and N-nitrosodimethylamine N-demethylation), NADPH-P450 reductase (80 and 400 nM, respectively), b_5 (80 and 400 nM, respectively), lipid mixture (20 μg/mL), sodium cholate (0.50 mM), and 0.5 mM chlorzoxazone, and 1.0 mM aniline and N-nitrosodimethylamine in 100 mM potassium phosphate buffer (pH 7.4) containing NADH or NADPH (1.0 mM). Data represent means ± range of duplicate determinations. Numbers in parentheses indicate percent of the activities.

droxylation, and N-nitrosodimethylamine N-demethylation by liver microsomes using NADH as an electron donor were found to be 41, 33, and 50%, respectively, of those catalyzed with NADPH. There were no marked increases in the NADPH-dependent oxidations of chlorzoxazone, aniline, and N-nitrosodimethylamine by liver microsomes, when NADH was also present in the systems.

DISCUSSION

In the present study we examined the roles of b_5 in the oxidation of 7-ethoxycoumarin by reconstituted monooxygenase systems containing P450 2E1 and by human liver microsomes. Substrates including chlorzoxazone, aniline, and N-nitrosodimethylamine, as well as 7-ethoxycoumarin, which are reported to be catalyzed mainly by P450 2E1 [3, 5, 19, 30, 31, 36, 37] were also used to determine further the roles of b_5 in reconstituted and liver microsomal systems. To understand the basis for electron transfer mechanisms from NADH and/or NADPH to P450 2E1, we used all of the electron transfer components including NADH-b_5 reductase, NADPH-P450 reductase, and b_5 in reconstituted monooxygenase systems containing P450 2E1 and determined the effects of antibodies raised against NADPH-P450 reductase and b_5 in the oxidations of 7-ethoxycoumarin by human liver microsomes. It should be mentioned that although the number of P450 molecules in intact liver microsomes has been reported to be about 20 times greater than that of NADH-b_5 reductase or NADPH-P450 reductase [38], we determined the drug-oxidation activities with about two molar excess of these reductases over P450 in the present reconstituted conditions.

In reconstituted systems containing NADH-b_5 reductase, b_5, and P450 2E1, rates of 7-ethoxycoumarin O-deethylation were found to be about 0.3 nmol product formed/min/nmol P450; the turnover number was one-fourth that obtained in systems where NADH-b_5 reductase was replaced by NADPH-P450 reductase. These results suggest that P450 2E1 accepts electrons from NADH-b_5 reductase via b_5, as observed in our previous studies using P450 3A4 in reconstituted testosterone 6β-hydroxylation systems [16, 17], and that NADPH-P450 reductase is more capable of reducing P450 2E1 when b_5 is present in the reaction mixture to act as an intermediary electron carrier. The findings that removal of b_5 from the reconstituted systems containing P450 2E1 caused drastic decreases in 7-ethoxycoumarin O-deethylation activities support the above suggestion.

We have shown previously that about 10% of the testosterone 6β-hydroxylation activity could be supplied through direct electron flow from NADPH-P450 reductase to P450 in reconstituted systems containing NADPH-P450 reductase, b_5, and P450 3A4, although the reaction is largely dependent upon the presence of b_5 [17]. A similar tendency was also noted in the present P450 2E1 systems for 7-ethoxycoumarin O-deethylation with the following lines of evidence. Omission of b_5 from reconstituted systems containing NADPH, NADPH-P450 reductase, b_5, and P450 2E1 caused a decrease to 10% of the 7-ethoxycoumarin O-deethylation activities obtained in a complete system. Similar patterns were also noted in systems containing

TABLE 5. Effects of NADH and NADPH on chlorzoxazone 6-hydroxylation, aniline p-hydroxylation, and N-nitrosodimethylamine N-demethylation by liver microsomes of human sample HL-11

Cofactor	Chlorzoxazone 6-hydroxylation	Aniline p-hydroxylation (nmol product formed/min/nmol P450)	N-Nitrosodimethylamine N-demethylation
NADH	2.54 ± 0.31 (41)	5.0 ± 0.22 (33)	2.98 ± 0.27 (50)
NADPH	6.17 ± 0.30 (100)	15.2 ± 0.55 (100)	5.96 ± 0.21 (100)
NADH + NADPH	6.89 ± 0.31 (112)	15.4 ± 0.99 (101)	5.93 ± 0.40 (99)

Methods for determination of chlorzoxazone 6-hydroxylation, aniline p-hydroxylation, and N-nitrosodimethylamine N-demethylation by liver microsomes of human sample HL-11 were described in Materials and Methods. Data represent means ± range of duplicate determinations. Numbers in parentheses indicate percent of activities.

NADH, NADPH-P450 reductase, b_5, and P450 2E1 in reconstituted systems, where about 10% of the 7-ethoxycoumarin O-deethylation activities was detected in the absence of b_5.

Divalent metal ions such as Mg^{2+} have been reported to affect testosterone 6β-hydroxylation and nifedipine oxidation activities catalyzed by P450 3A4 in reconstituted systems [14]. One of the mechanisms underlying stimulation of these oxidation activities by Mg^{2+} is considered to involve accelerated electron flow from NADPH-P450 reductase to b_5, and thus the first electron flow from NADPH-P450 reductase to P450 3A4 could be accelerated [16]. In contrast to the P450 3A4 systems, P450 2E1-catalyzed 7-ethoxycoumarin O-deethylation in reconstituted systems was not dependent upon the presence of Mg^{2+} in a potassium phosphate buffer but was partially dependent upon Mg^{2+} in a potassium HEPES buffer. The different tendency for the requirement of GSH between P450 3A4- and P450 2E1-catalytic activities was also noted where GSH could accelerate 7-ethoxycoumarin O-deethylation by P450 2E1 only in the potassium HEPES buffer. These results suggest that there might be different mechanisms of stimulation by b_5 in the drug-oxidation reactions catalyzed by P450 3A4 and 2E1 in reconstituted systems.

An interesting finding of the present studies was that NADH could serve as an effective electron donor for 7-ethoxycoumarin O-deethylation catalyzed by reconstituted P450 2E1 systems containing NADPH-P450 reductase and b_5, and by liver microsomes from human sample HL-11 which contains relatively high levels of P450 2E1 [19]. This effect was also observed in reconstituted P450 2E1 and liver microsomal systems using as substrates chlorzoxazone, aniline, and *N*-nitrosodimethylamine, which have been reported to be oxidized mainly by P450 2E1 in experimental animals and humans [3, 5, 19, 30, 31, 36, 37]. When other P450 enzymes including recombinant human P450s 1A1 and 1A2 and native rat P450s 1A1, 1A2, and 2B1 were used, NADH played minor roles in the reconstituted 7-ethoxycoumarin O-deethylation activities. Our previous studies have also suggested that NADH is a poor electron donor for oxidations of testosterone and nifedipine catalyzed by P450 3A4 in reconstituted and liver microsomal systems [17]. These results suggest that the roles of b_5 in P450 2E1-catalyzed drug-oxidation reactions might be different from those catalyzed by other P450 enzymes; further work will be necessary to explain these differences. It is also interesting to note that there were no marked increases in P450 2E1-catalyzed oxidations of 7-ethoxycoumarin, chlorzoxazone, aniline, and *N*-nitrosodimethylamine by human liver microsomes when NADH was added to the NADPH-supported systems, although these substrates required b_5 to exert full activities in reconstituted P450 2E1 systems.

Patten and Koch [39] have reported recently that addition of b_5 to Sf9 insect cell systems expressing human P450 2E1 using baculovirus vector causes decreases in the rate of NADPH utilization and H_2O_2 formation, and thus the product formation could be accelerated with apparent increases in reducing equivalents for P450 2E1-catalytic reactions. Similar mechanisms for the roles of b_5 in reconstituted monooxygenase systems containing P450 3A4 [40, 41] and rabbit P450 2B4 [42] have also been proposed. The question arises as to why there are similar mechanisms for the effect of b_5 in enhancement of the drug-oxidation activities catalyzed by different forms of P450, despite the fact that responses to Mg^{2+} and GSH in 7-ethoxycoumarin O-deethylation in reconstituted P450 2E1 systems differed from testosterone 6β-hydroxylation catalyzed by P450 3A4 reconstituted system [16, 17].

In this study we used anti-b_5 and anti-NADPH-P450 reductase antibodies to examine the effects of electron transfer components on the 7-ethoxycoumarin O-deethylation activities catalyzed by human liver microsomes. It was found that anti-NADPH-P450 reductase IgG inhibited the 7-ethoxycoumarin O-deethylation more strongly in NADPH- than NADH-supported system by liver microsomes, although anti-b_5 IgG inhibited both NADH- and NADPH-supported 7-ethoxycoumarin O-deethylation activities to similar extents. Similar approaches have also been reported by Kuwahara and Mannering [43] who showed that b_5 is an absolute requirement for the NADH-supported *p*-nitrophenetole O-deethylation activities by rat liver microsomes.

Four different P450 2E1 substrates were examined in this study. The relevance of these findings to other substrates and details of the mechanism of b_5 stimulation are the subject of further investigation.

This work was supported, in part, by Grants from the Ministry of Education, Science, and Culture of Japan, the Ministry of Health and Welfare of Japan, the Developmental and Creative Studies from the Osaka Prefectural Government, and by United States Public Health Service Grants CA44353 and ES00267.

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