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COVALENT BINDING OF ^{14}C- AND ^{35}S-LABELED THIOCARBAMIDES IN RAT HEPATIC MICROSOMES

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Abstract—The covalent binding of a series of ^{14}C- or ^{35}S-labeled benzimidazole-2-thione (MBI) derivatives to rat liver microsomal proteins was studied to determine the mechanisms of hepatic monooxygenase oxidation of model anti-hyperthyroid compounds. All thiocarbamides tested (including methimazole) produced an NADPH-dependent loss of cytochrome P450 (P450) chromophore which could be prevented by the addition of glutathione (GSH). The covalent binding of MBI to liver microsomal proteins from dexamethasone (DEX)-pretreated rats was enhanced 10-fold with NADPH, unaffected by P450 inactivation with 1-aminobenzotriazole (ABT) and attenuated by GSH addition. Heat treatment of microsomes to inactivate the flavin-containing monooxygenase (FMO) decreased the observed binding. Equivalent amounts of [^{35}S]- and [^{14}C]MBI were covalently bound to hepatic microsomal proteins, suggesting retention of both the carbon and sulfur portions of the molecule in the MBI/protein adduct. Thiophilic reagents effected release of covalently bound [^{14}C]- and [^{35}S]MBI in equal amounts suggesting the presence of disulfide bonds between an MBI-derived sulfenic acid and microsomal protein thiols. Coincubation with bovine serum albumin (BSA) resulted in NADPH-dependent binding of [^{14}C]-MBI to BSA sulfhydryls which was blocked by prior treatment of BSA with iodoacetamide. 1-Methylbenzimidazole-2-thione (MMBI) also covalently bound to microsomal proteins and BSA but at levels lower than with MBI. P450, however, appeared to be more important than FMO in the metabolism of MMBI based on the effects of microsome heat pretreatment or ABT addition. In addition, *ca.* 1.5-fold more ^{35}S- than ^{14}C-label became bound. The covalent binding of [^{35}S]1,3-dimethyl-benzimidazole-2-thione (DMMBI) to microsomal proteins was *ca.* six times greater than that of [^{14}C]DMMBI. ABT, catalase and superoxide dismutase had a minimal effect on [^{35}S]DMMBI binding, while FMO inactivation decreased binding by *ca.* 30%. These findings suggest that both monooxygenases contribute significantly to the hepatic metabolism of thiocarbamides. However, FMO activates thiocarbamides primarily to sulfenic acids, whereas P450 appears to produce both sulfenic acid and other reactive sulfur-derived metabolites. Thiol groups of P450 and other proteins are the molecular targets for these reactive species formed during the hepatic metabolism of anti-hyperthyroid drugs.

Many organosulfur compounds are activated by hepatic microsomal monooxygenases [cytochrome P450 (P450) isozymes or the flavin-containing monooxygenase (FMO†)] to reactive species which may covalently bind to protein, deplete hepatic glutathione (GSH) levels, inactivate P450 isozymes, and/or produce hepatic necrosis [1, 2]. P450 alone metabolizes carbon disulfide or parathion to species that produce covalent sulfur binding to proteins [3, 4]. However, methimazole (MMI), an important drug used in anti-hyperthyroid therapy, is metabolized by both hepatic microsomal monooxygenases to species which inactivate P450 isozymes [5–8]. P450 effects differential binding of the carbon and sulfur fragments of MMI to P450 apoprotein wherein 70% of the binding is due to the sulfur atom alone [6]. FMO is the predominant hepatic microsomal catalyst in the metabolic activation of the ethylene-bis-dithio-carbamate fungicide metabolite, ETU, although some P450 isozymes also appear to contribute [9]. Microsomal metabolism of ETU causes covalent binding of [^{14}C]ETU to microsomal proteins, elevation of oxidized glutathione (GSSG) levels in incubations supplemented with GSH, and inactivation of P450 isozymes by covalent binding to the apoprotein. The present study uses three pairs of ^{14}C- and ^{35}S-labeled thiocarbamides to determine the chemical and enzymatic mechanisms of activation and covalent protein binding in rat hepatic microsomal systems for a series of model anti-hyperthyroid agents.

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† Abbreviations: ABT, 1-aminobenzotriazole; BHT, butylated hydroxytoluene; BNF, β-naphthoflavone; BSA, bovine serum albumin; DEX, dexamethasone; CN^-, cyanide ion; DMMBI, 1,3-dimethyl-benzimidazole-2-thione; DTT, dithiothreitol; ETU, ethylenethiourea; FMO, flavin-containing monooxygenase; GSH, glutathione; GSSG, oxidized glutathione; LSC, liquid scintillation counting; MBI, benzimidazole-2-thione; MMBI, 1-methyl-benzimidazole-2-thione; MMI, methimazole; and PB, phenobarbital.

MATERIALS AND METHODS

Chemicals. GSH, dexamethasone (DEX), β-naphthoflavone (BNF), phenobarbital (PB), dithiothreitol (DTT), GSSG, NADPH, catalase, and superoxide dismutase were purchased from the Sigma Chemical Co., St. Louis, MO. 1-Aminobenzotriazole (ABT) was synthesized as described in Ref. 9. [^{14}C]-Benzimidazole-2-thione (MBI), [^{35}S]MBI, [^{14}C]1-methyl-benzimidazole-2-thione (MMBI), and [^{35}S]-MMBI were synthesized as described in Ref. 10. Radiolabeled 1,3-dimethyl-benzimidazole-2-thione (DMMBI) was synthesized by adaptation of literature

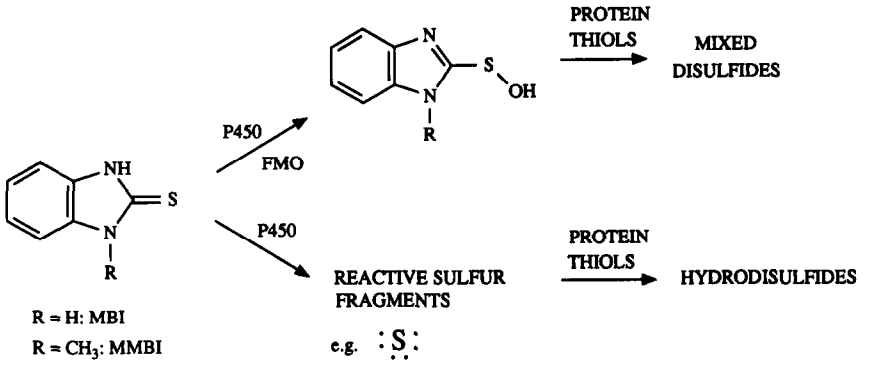

Fig. 1. Microsomal monooxygenase-mediated activation of thiocarbamides.

methods [10, 11] and purity was determined by HPLC (65% acetonitrile/water, Novapak C18, Waters Associates, Milford, MA). Specific activities, determined by liquid scintillation counting (LSC) and HPLC, were: [^{14}C]MBI (2.2 mCi/mmol), [^{35}S]MBI (0.35 mCi/mmol), [^{14}C]MMBI (3.0 mCi/mmol), [^{35}S]MMBI (1.37 mCi/mmol), [^{14}C]DMMBI (0.54 mCi/mmol), and [^{35}S]DMMBI (1.84 mCi/mmol).

Animals and microsomal preparation. Male Sprague–Dawley rats (220–250 g) were obtained from Bantin Kingman Laboratories, Gilroy, CA, and were left untreated or were pretreated with PB, BNF or DEX [9]. Microsomes were prepared as described in Ref. 9.

Biochemical assays. P450 chromophore of microsomal incubations (conducted as described below but containing 1.0 mM [^{12}C]thiocarbamide) was measured by the method of Estabrook *et al.* [12] using a Perkin–Elmer Lambda 5 scanning spectrophotometer.

Covalent binding of thiocarbamide compounds to microsomal proteins was determined in 2.0-mL incubations containing 1.0 mM (0.36 μCi) thiocarbamide (or 0.5 mM DMMBI), 1.5 mM diethylaminetriaminopentaacetic acid, and 1–2 mg/mL microsomal protein with or without 1.0 mM NADPH in 0.1 M phosphate buffer, pH 7.4. Microsomes preincubated at 37° for 1 hr were used in some incubations while in others either 3.0 mM ABT, 5.0 mM GSH, 4.5 mg/mL bovine serum albumin (BSA) or 4.5 mg/mL iodoacetamide-treated BSA was included. After a 15-min incubation at 37°, the assay was terminated by the addition of 15 mL of 5% H_2SO_4 in methanol, or by chilling in ice. For some experiments GSSG (5 mM) or a thiophilic agent [cyanide ion (CN^-) (20 mM), GSH (5 mM), or DTT (20 mM)] was added to chilled incubations and left for 3 hr before precipitation of protein with H_2SO_4/methanol. Protein pellets were washed at least six times with 3.0 mL methanol. Further washing did not remove additional radioactivity from the samples. The protein pellet was dissolved in 0.75 mL of 1.0 N NaOH, and aliquots were subjected to LSC or protein determination [13].

BSA (30 mg/mL) was treated with 7 mM iodoacetamide for 15 min. The protein was then dialyzed extensively to remove unbound iodoacetamide. Accessible sulfhydryls before and after iodoacetamide treatment were quantified using Ellman's reagent with GSH as a reference [14] (0.41 nmol thiol/nmol protein for native BSA; 0.03 nmol thiol/nmol protein for iodoacetamide-treated BSA). Incubations containing BSA or iodoacetamide-treated BSA were terminated by chilling on ice, and then centrifuged at 105,000 *g* for 30 min to isolate the microsomal and supernatant fractions which were added to 15 mL of 5% H_2SO_4 in methanol for covalent binding determinations as detailed above.

RESULTS

Chromophore studies. The NADPH-dependent effects of MBI and analogs (Fig. 1) were assessed *in vitro* using hepatic microsomes from untreated and PB-, BNF-, or DEX-pretreated rats to examine whether these compounds were able to destroy P450 chromophore as was observed with ETU [9]. The different induction states were chosen to assess P450 isozyme [15] selectivity. P450 enzymatic activity was not monitored for all compounds due to their tendency to partition with the microsomal fraction and competitively inhibit P450 activity. An NADPH-dependent decrease in P450 chromophore was observed for all thiono-compounds tested (Fig. 2); this decrease could be blocked by the addition of GSH at physiological concentrations (data not shown). Loss of P450 chromophore was usually accompanied by an increase in P420, the form of P450 possessing an altered cysteine-heme iron ligand. P450 isozymes inducible by PB were less susceptible to thiocarbamide-mediated P450 chromophore loss than constitutive, BNF- or DEX-inducible P450 isozymes, and the extent of P450 chromophore loss was approximately equal for all compounds within each microsomal system (Fig. 2). MMI produced losses of P450 chromophore in all microsomal systems (data not shown) comparable to those shown in Fig. 2.

Covalent binding to microsomal protein. The covalent binding of MBI to hepatic microsomal protein from DEX-pretreated rats was enhanced 10-

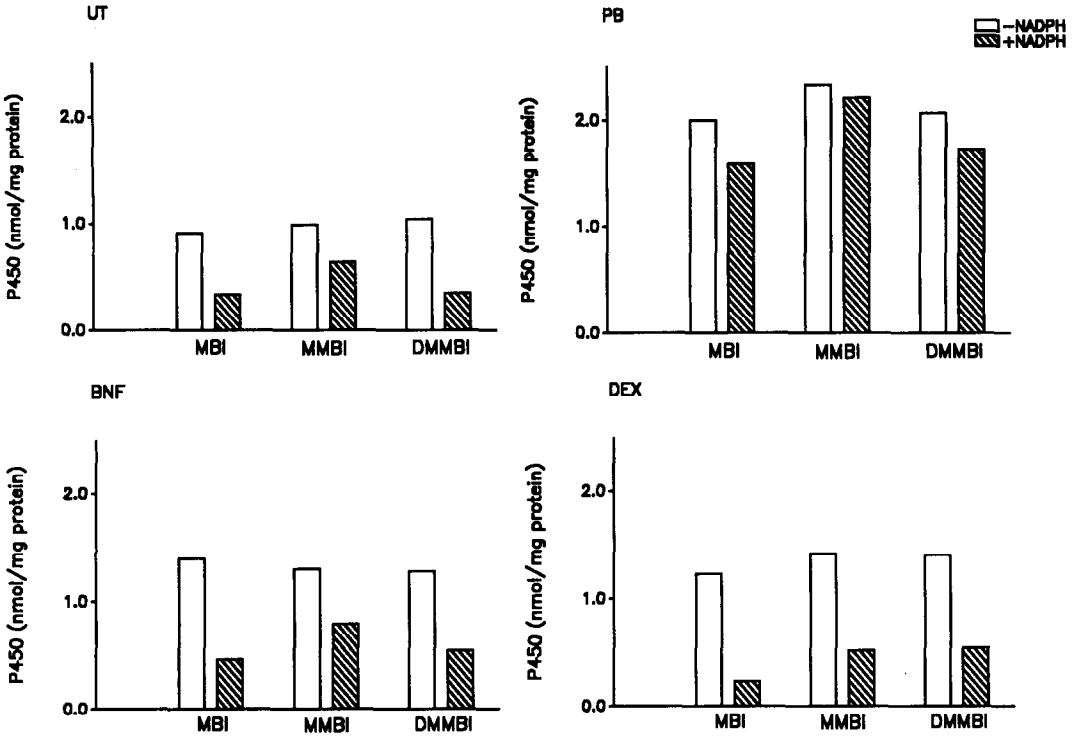

Fig. 2. Thiocarbamide-mediated P450 chromophore loss in microsomes from untreated (UT) and PB-, BNF- or DEX-pretreated rats. Incubations were conducted as described in Materials and Methods. Values are the means of two individual determinations from one experiment.

fold in incubations containing NADPH (Fig. 3). This binding was abolished by coincubation with GSH, decreased by heat treatment of microsomes, and unaffected by the addition of the P450 suicide substrate ABT. Under these conditions, heat pretreatment of microsomes caused a >95% loss of FMO activity, with minimal effects on most P450 activities [9]. Equivalent amounts of [^{35}S]- and [^{14}C]-MBI bound covalently to microsomal proteins. The [^{14}C]/[^{35}S]MBI covalent binding ratio was also 1:1 at a higher MBI concentration (10 mM). Covalent binding at this MBI concentration was similarly attenuated by heat pretreatment of DEX-pretreated rat microsomes (data not shown). The binding patterns of [^{35}S]- and [^{14}C]MBI to microsomal proteins from untreated rats were similar to those with DEX-pretreated rat microsomal protein, except that FMO inactivation produced a larger attenuation in binding (Fig. 3).

Addition of GSSG after the covalent binding of MBI to microsomal proteins from DEX-pretreated rats resulted in no release of bound MBI [i.e. covalent binding levels were equivalent to those observed without GSSG addition (Figs. 4 and 3)]. Addition of DTT removed covalently bound MBI to levels comparable to those observed in the absence of NADPH (Fig. 4). GSH and CN^- also removed the majority of the bound MBI. Similar results were obtained when microsomes from untreated rats were used (data not shown).

The covalent binding of [^{35}S]MMBI to microsomal proteins of DEX-pretreated rats was *ca.* 1.5-fold greater than that observed for the ^{14}C-labeled analog (Fig. 5), and was lower than that observed with MBI (Fig. 3). Coincubation with GSH blocked all [^{14}C]- and [^{35}S]MMBI covalent binding, as observed in studies employing MBI. Heat treatment of microsomes did not affect the binding of either [^{14}C]- or [^{35}S]MMBI to microsomal proteins. However, ABT inclusion decreased the covalent binding of [^{35}S]-MMBI to proteins significantly and that of [^{14}C]-MMBI slightly. Incubation of microsomes with ABT and NADPH for 10 min prior to addition of [^{35}S]-MMBI (to eliminate possible competition between ABT and MMBI) had similar effects on [^{35}S]MMBI binding (data not shown). Similar results also were obtained when untreated rat microsomes were used (data not shown).

The N,N-disubstituted analog DMMBI produced losses in both P450 chromophore and enzymatic activity (data not shown). The [^{14}C]- or [^{35}S]DMMBI covalently bound to microsomal proteins from DEX-pretreated rats was approximately 3- to 4-fold greater in the presence of NADPH (Fig. 6). ABT, catalase and superoxide dismutase had little effect on [^{35}S]-DMMBI binding (data not shown), while heat pretreatment of microsomes decreased binding by *ca.* 30%. Preincubation of microsomes with ABT and NADPH prior to [^{35}S]DMMBI addition also produced no attenuation in covalent binding (data not shown). NADPH-dependent and -independent binding of [^{14}C]DMMBI to microsomal proteins was

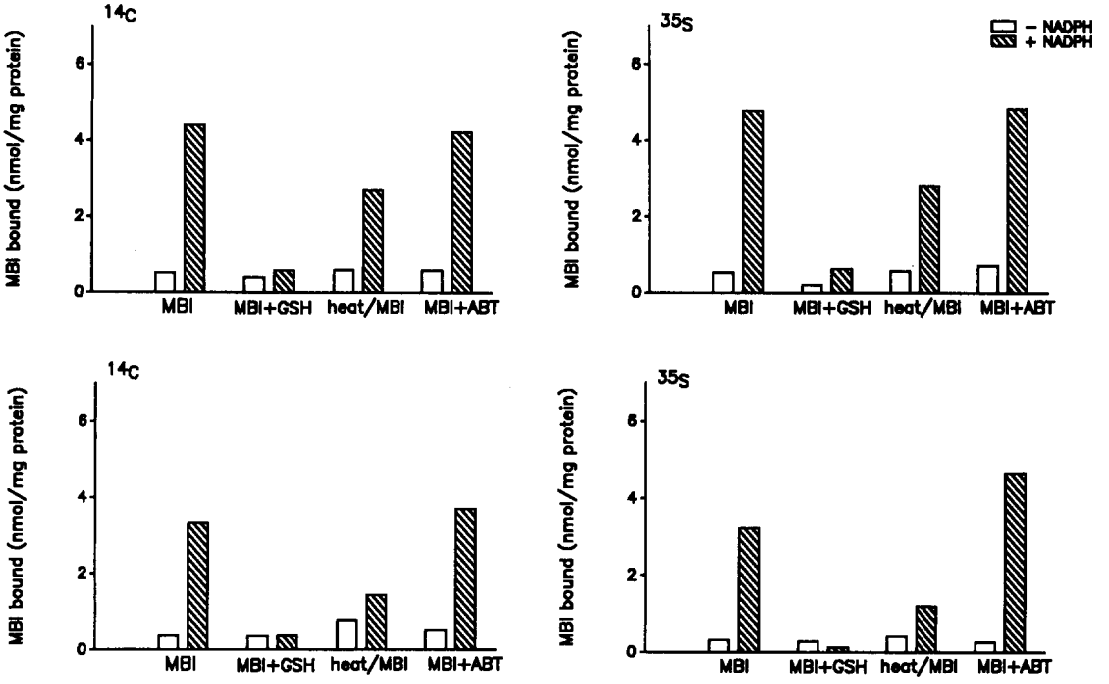

Fig. 3. Covalent binding of [^{14}C]- or [^{35}S]MBI to microsomal proteins of DEX-pretreated or untreated rats. Microsomal protein was obtained from DEX-pretreated rats (upper panels) and untreated rats (lower panels). Incubations were performed as described in Materials and Methods. Key: +GSH: 5.0 mM GSH or +ABT: 3.0 mM ABT was included in the incubation; heat/MBI: prior to use, microsomes were incubated at 37° for 1 hr to inactivate FMO. All values are the means of two individual determinations from one experiment.

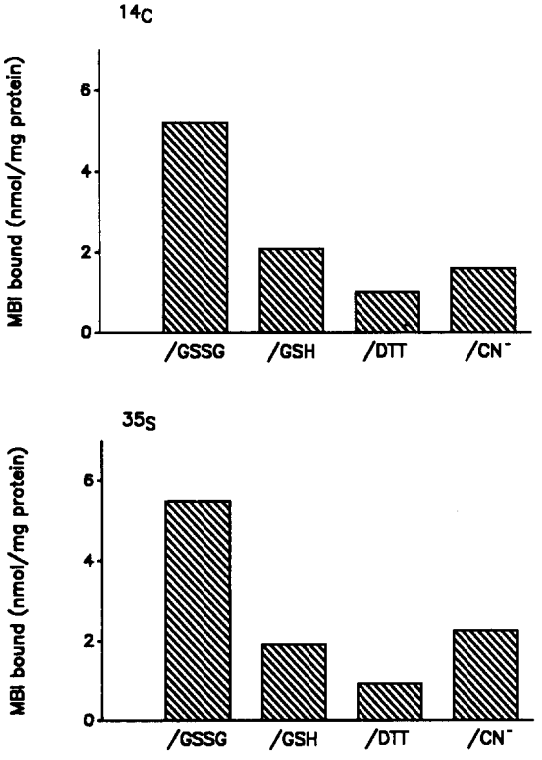

Fig. 4. Release of [^{14}C]- or [^{35}S]MBI from DEX-pretreated rat microsomal proteins by thiophilic reagents. Incubations (+NADPH) received GSSG, GSH, DTT or CN^- as described in Materials and Methods. Values are the means of two individual determinations from one experiment.

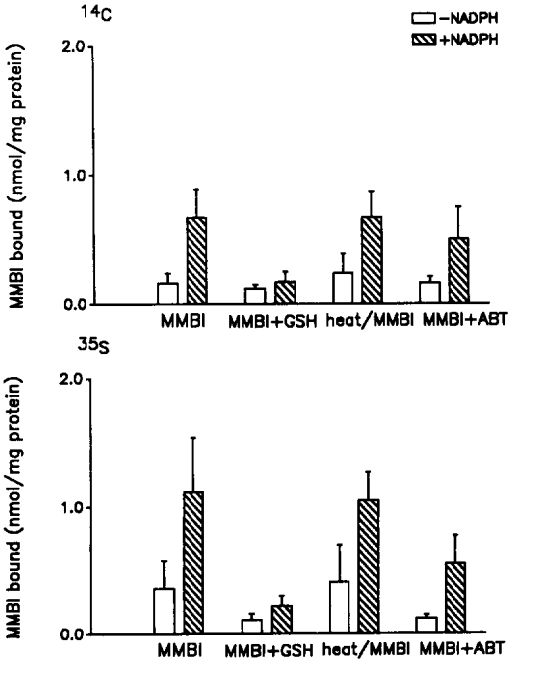

Fig. 5. Covalent binding of [^{14}C]- or [^{35}S]MMBI to microsomal proteins of DEX-pretreated rats. Incubation conditions were the same as for Fig. 3. Values are the means ± SD of determinations from four separate experiments.

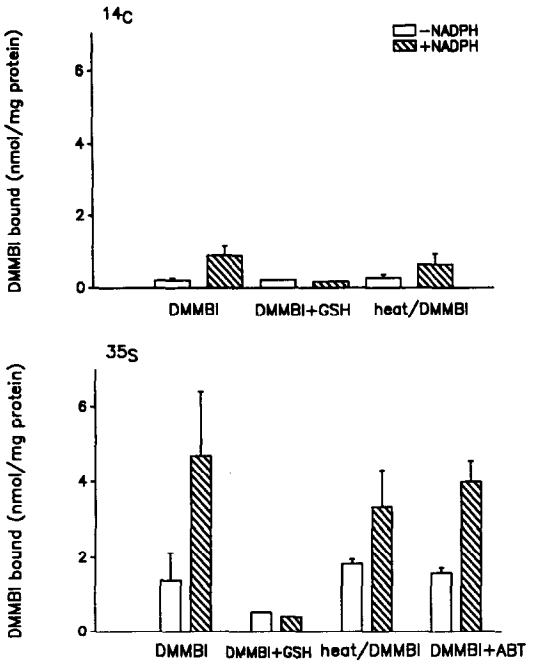

Fig. 6. Covalent binding of [^{14}C]- or [^{35}S]DMMBI to microsomal proteins of DEX-pretreated rats. Incubation conditions were the same as for Fig. 3. Values are the means ± SD of determinations from three separate experiments, except for the GSH data which represent a mean of two determinations from one experiment.

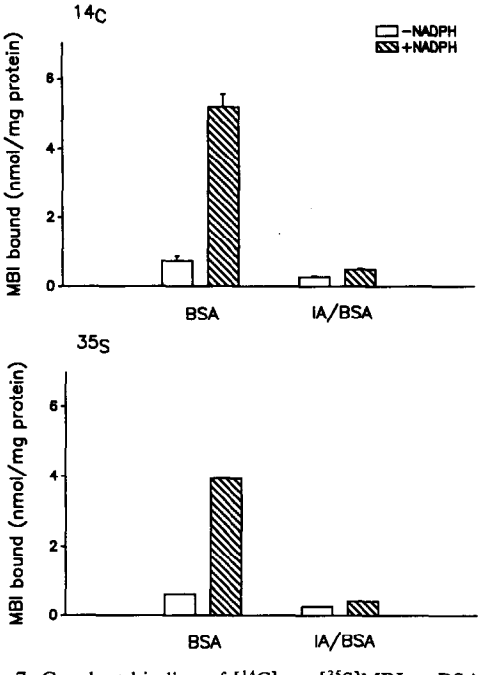

Fig. 7. Covalent binding of [^{14}C]- or [^{35}S]MBI to BSA or acetamidated BSA. Incubations were conducted as described in Materials and Methods using DEX-pretreated rat microsomes. BSA: BSA; or IA/BSA: iodoacetamide-treated BSA was added to the incubation. Values for [^{14}C]-MBI are the means ± SD of three determinations from one experiment and for [^{35}S]MBI, the mean of two determinations from one experiment.

approximately 6-fold less than that of [^{35}S]DMMBI (Fig. 6). DTT removed 70% of covalently bound [^{35}S]-DMMBI (relative to samples incubated with GSSG), while GSH (15%) and CN^- (12%) were much less effective (data not shown).

Covalent binding to BSA. Native BSA or iodoacetamide-treated BSA was added to microsomal incubations as an alternate electrophile scavenger. BSA was separated from the microsomal fraction by centrifugation of the incubation mixtures. The covalent binding of [^{14}C]- or [^{35}S]MBI to native BSA which occurred in the presence of NADPH was blocked by pretreatment of BSA with iodoacetamide (Fig. 7). Under these conditions, approximately 85% of the free thiol groups were bound with MBI. Covalent binding of [^{14}C]- or [^{35}S]-MBI to the microsomal fraction was decreased only slightly by the addition of BSA or iodoacetamide-treated BSA (data not shown).

The binding of MMBI and DMMBI to BSA or iodoacetamide-treated BSA was also assessed using DEX-pretreated rat microsomes (N = 2, for one experiment). [^{35}S]MMBI bound covalently to BSA (−NADPH: 0.23 nmol/mg protein; +NADPH: 0.74 nmol/mg protein) at levels lower than those observed with microsomal protein (Fig. 5). Almost no covalent binding of [^{35}S]MMBI to iodoacetamide-treated BSA occurred (−NADPH: 0.25 nmol/mg protein; +NADPH: 0.33 nmol/mg protein). [^{35}S]-DMMBI also bound covalently to BSA (−NADPH: 1.42 nmol/mg protein; +NADPH: 2.44 nmol/mg protein). Again, this binding was eliminated when iodoacetamide-treated BSA was used (−NADPH: 0.67 nmol/mg protein; +NADPH: 0.68 nmol/mg protein).

DISCUSSION

The effects of thiocarbamide compounds on the P450 chromophore of liver microsomes obtained from untreated and PB-, BNF- or DEX-pretreated rats (Fig. 2) mirrored those previously observed with ETU [9]. These studies indicated that hepatic monooxygenases (P450 or FMO) oxidize these thiocarbamide compounds to diffusible species which cause the conversion of P450 to P420 and inactivate P450 isozymes, presumably by binding to the apoprotein as previously described for ETU [9]. All compounds tested effected similar extents of P450 chromophore loss within each microsomal system (Fig. 2). P450 isozymes inducible by PB were less susceptible, as previously observed for ETU-mediated P450 chromophore and activity loss [9]. Although P450 isozymes inducible by DEX were inactivated markedly by the thiocarbamides tested, P420 formation was not as pronounced as that observed in microsomes obtained from untreated or BNF-pretreated rats. Previous studies with the thiol blocking reagent mersalyl demonstrated that the order of susceptibility in the conversion of P450 to

P420 was BNF-inducible > uninduced > PB-inducible rat or rabbit hepatic P450 isozymes [16]. These findings suggest that the primary and tertiary structures of P450 isozymes confer susceptibility to P420 formation through the accessibility of sulfhydryl groups to thiol modifying reagents such as mercurials or thiocarbamide metabolites. The number of cysteine residues of several rat hepatic P450 isozymes correlates with these observations as P450IA1 (BNF-inducible) possesses eight cysteine residues; P450IICl1 (constitutive, male-specific) 7; P450IIIA1 (DEX-inducible) 6; and P450IIB1 (PB-inducible) 5 [17, 18].

To ascertain directly whether protein sulfhydryls are the site of thiocarbamide binding, native bovine serum albumin or iodoactamide-treated BSA was added to microsomal incubations as an alternate electrophile scavenger. The observation that [^{14}C]- or [^{35}S]MBI (Fig. 7), [^{35}S]MMBI and [^{35}S]DMMBI covalently bound to BSA but not to iodoacetamide-treated BSA unequivocally indicates that free protein thiols are molecular targets for electrophilic thiocarbamide metabolites as suggested in previous studies [9].

The binding of [^{14}C]MBI to microsomal proteins of untreated or DEX-pretreated rats (Fig. 3) paralleled findings obtained with ETU [9]. The effects of heat pretreatment or ABT addition strongly suggest that FMO is the primary enzyme for metabolic activation of MBI to species which bind covalently to microsomal proteins, although P450 isozymes of the IIIA subfamily (DEX-inducible) also appear to be involved in the absence of FMO. The complete blockade of such covalent binding by GSH addition indicates that the reactive intermediate(s) which binds to microsomal proteins is diffusible, as previously observed for ETU [9]. The identical covalent binding pattern for [^{14}C]- and [^{35}S]MBI suggests that singlet atomic sulfur or other sulfur-containing fragments are *not* produced as a reactive metabolite under the conditions of these experiments (see Fig. 1). The similarities in microsomal metabolism of MBI and ETU suggest that [^{35}S]MBI is a useful model for the reactions of [^{35}S]ETU, a compound not readily available.

These results contrast with those previously obtained for MMI where [^{35}S] binding to microsomal proteins was *ca.* 3-fold greater than [^{14}C] binding [6]. Lee and Neal found that high levels of MMI are required to saturate PB-pretreated rat microsomes (K_m = *ca.* 20 mM) [6]. However, the lack of any increased binding in the present study when conducted at a higher MBI concentration (under conditions where the contributions of FMO were mimimal) is not consistent with a lower affinity P450-dependent process. In addition, the same [^{14}C]/[^{35}S] binding ratio observed at high and low MBI concentrations suggests that the same enzymatic activation mechanism is operating. The lower concentration used in this study (1 mM) should be sufficient to saturate FMO since K_m values for most thiocarbamides are generally in the low micromolar range [2, 19].

Addition of the nucleophile DTT after covalent binding of ETU to microsomal proteins resulted in the complete release of bound ETU [9]. DTT, GSH, and CN^- were thus evaluated to determine the nucleophilic and steric requirements for release of covalently bound MBI from microsomal proteins. The finding that GSSG did not release bound MBI from microsomal proteins indicates that a free thiol function is required for MBI release. All thiophilic reagents used (DTT, GSH and CN^-) removed almost identical percentages of bound [^{14}C]- and [^{35}S]MBI, suggesting release of the entire MBI molecule from microsomal protein sulfhydryl groups. If binding of atomic sulfur to form hydrodisulfides were a significant outcome, excess [^{35}S] release by CN^- would have resulted [1, 6]. GSH was equivalent to DTT in the ability to remove ETU from microsomal proteins (>90% [9]) but was less sufficient in removing MBI, suggesting a different in the accessibility of the large GSH molecule to the protein adducts of ETU vs MBI.

MMBI had similar effects on P450 chromophore and enzymatic activity (data not shown), but the binding to microsomal proteins was lower than that for MBI. Since heat treatment of microsomes had no effect on MMBI binding and ABT significantly decreased [^{35}S]MMBI binding, it is concluded that P450, rather than FMO, is predominant in the activation of this compound to species which binds to proteins. These results distinguish MMBI from ETU and MBI for which FMO is the predominant microsomal catalyst. It is therefore likely that fewer reactive metabolites of MMBI are produced, compared to either ETU or MBI, because the primary catalyst responsible for MMBI activation (i.e. P450) is inactivated during the metabolism of this compound. It was determined that FMO, the primary catalyst for ETU and MBI oxygenation, is not dramatically inactivated as a consequence of ETU metabolism [9]. It is also possible that the production of reactive species in the P450 active site makes binding to critical residues more likely than when produced externally by FMO. Different reactivities of MBI and MMBI metabolites with protein nucleophiles may also contribute to the observed differences in covalent binding. Since GSH addition attenuated all covalent binding of MMBI, the reactive MMBI intermediates produced by P450 are diffusible, as observed for both MBI (Fig. 3) and ETU [9].

The greater binding of [^{35}S]MMBI than [^{14}C]-MMBI is consistent with the P450-catalyzed formation and subsequent binding of singlet atomic sulfur, the putative intermediate formed during microsomal metabolism of CS_2 [4], parathion [3] and MMI [6]. However, these data cannot exclude the formation and binding of other reactive sulfur-containing fragments. It should also be noted that the majority of bound MMBI contains *both* carbon and sulfur portions of the molecule which is consistent with the binding of a sulfenic acid metabolite (see Fig. 1). N-Methylation of MBI appears to cause a switch in enzyme selectivity from FMO with MBI to P450 with MMBI. The molecular basis for this selectivity switch is unknown since the substrate specificites of FMO and P450 are not typically affected by such modest steric or polarity modifications.

Microsomal metabolism of DMMBI also resulted in NADPH-elicited covalent binding to microsomal

proteins with a ratio of [^{35}S]/[^{14}C]DMMBI bound of *ca.* 6 (Fig. 5). The excess in [^{35}S] binding over [^{14}C] from DMMBI, both in the presence and absence of NADPH, suggests that the sulfur portion of the molecule can be released by monooxygenase-dependent and -independent metabolism. The *ca.* 30% decrease in covalent binding caused by heat pretreatment suggests that FMO (or another heat-sensitive process) may be involved in the covalent binding of DMMBI to microsomal proteins, although not to the extent that it is in the case of MBI or ETU. Since ABT had little effect on this binding, P450 does not appear to be the catalyst. The lack of effect upon addition of catalase, superoxide dismutase or butylated hydroxytoluene (BHT) to microsomal incubations suggest that reactive oxygen species are also not involved in the covalent binding of [^{35}S]DMMBI to microsomal proteins. The finding that GSH abolished essentially all NADPH-dependent binding again suggests the formation of a diffusible intermediate that is capable of reacting with thiols.

The release of bound DMMBI from microsomal proteins by DTT but not CN^- or GSH treatment also distinguishes it from all other thiocarbamides tested. This sulfur binding is, therefore, not consistent with hydrodisulfide formation which was proposed previously as a result of singlet atomic sulfur addition to free thiol groups [3, 4, 6]. These previous studies employed CN^- to effect diminution of [^{35}S] binding and concomitant formation of thiocyanate ion. The contrasting results with DMMBI could result from different sites of sulfur binding with attendant differences in reactivity toward CN^-. The low [^{14}C]/[^{35}S]DMMBI binding ratio also suggests that the binding of a sulfenic acid species is not important. Moreover, the formation of such a metabolite from DMMBI is precluded by the absence of tautomeric protons which are present in MBI and MMBI.

Both [^{14}C]- and [^{35}S]MBI bound covalently to BSA and to microsomal proteins at similar levels (Figs. 3 and 7), whereas [^{35}S]MMBI and [^{35}S]-DMMBI binding to BSA was lower than to microsomal proteins. Thus, it appears that there is a preference for MMBI- and DMMBI-derived reactive metabolites to bind to microsomal protein rather than to BSA, whereas no target preference exists for MBI. The production of reactive MMBI metabolites by P450 may facilitate binding to nearby residues of the apoprotein, thus offering a partial explanation for the target specificity of MMBI, if not DMMBI.

These studies indicate that during the hepatic microsomal metabolism of MBI (and by inference ETU) atomic sulfur production and subsequent covalent binding to microsomal proteins does *not* occur. Since these oxygenations proceed exclusively by FMO catalysis, P450 involvement appears to be required for the production of reactive sulfur-derived fragment metabolites like singlet atomic sulfur. These data also support the hypothesis that microsomal activation of many thiocarbamide compounds *by either FMO or P450* proceeds via sulfenic acid species that react with protein thiols to form adducts which retain both carbon and sulfur portions of the molecule. These findings are consistent with the role of FMO in the metabolic conversion of thiocarbamides to sulfenic acids [2, 19]. P450-dependent metabolism was significant in the case of MMBI, and the observed binding pattern suggests that both sulfur oxidation mechanisms (i.e. reactive sulfur fragment release and sulfenic acid production) can be catalyzed by P450. These studies provide unique examples of structural requirements governing the thiocarbamide substrate specificities of FMO and P450, which may aid in assessing the potential hepatic toxicity of anti-hyperthyroid drugs.

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