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S-Methyl *N*-Butylthiocarbamate Sulfoxide

SELECTIVE CARBAMOYLATING AGENT FOR MOUSE MITOCHONDRIAL ALDEHYDE DEHYDROGENASE

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ABSTRACT. Liver mitochondrial low-K_m aldehyde dehydrogenase (ALDH2, EC 1.2.1.3), the isoform responsible for the conversion of acetaldehyde to acetate, is inhibited by the sulfoxide bioactivation products of $Et_2NC(O)SMe$ (from the alcohol aversion drug disulfiram), $Pr_2NC(O)SEt$ (the herbicide *S*-ethyl *N*,*N*-dipropylthiocarbamate), and BuNHC(O)SMe (from the fungicide benomyl). This study tested the hypothesis that bioactivated BuNHC(O)SMe, the most potent of these thiocarbamates, is a selective carbamoylating agent for ALDH2 of mouse liver *in vivo* and *in vitro*. [^{14}C]BuNHC(O)SMe administered i.p. to mice labeled one principal mitochondrial protein, which cochromatographed with ALDH activity by in-gel assay after isoelectric focusing. The labeled protein was isolated by isoelectric focusing (pI 6.1) and SDS–PAGE (54 kDa) and identified as ALDH2 by sequencing of peptides from a tryptic digest. *In vivo* at 1.5 mg/kg, enzyme inhibition was 80%, and ALDH2 was the only mitochondrial protein labeled extensively, illustrating the outstanding potency and specificity. ALDH2 also was labeled upon incubation of mouse liver mitochondria with [^{14}C]BuNHC(O)SMe in the presence of microsomes (P450) and NADPH. In contrast, under similar conditions, [^{14}C]$Pr_2NC(O)SEt$ sulfoxide labeled primarily two other proteins at ~58 and ~61 kDa, establishing a very different selectivity for the two sulfoxides. These findings are of interest relative to selective inhibitors and carbamoylating agents for ALDH2 and to alcohol aversion upon exposure to herbicides and fungicides. BIOCHEM PHARMACOL **58**;9:1467–1473, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. aldehyde dehydrogenase; enzyme inhibition; carbamoylation; thiocarbamate sulfoxide; EPTC; disulfiram; benomyl

The alcohol aversion drug disulfiram, via its metabolite $Et_2NC(O)SMe$, and the herbicide EPTC (two *S*-alkyl *N*,*N*-dialkylthiocarbamates) inhibit ALDH2† (EC 1.2.1.3) following bioactivation to the corresponding sulfoxides [1–3] (Fig. 1). *N*-Depropyl EPTC (an EPTC metabolite [4, 5]) and MBT (a metabolite of the fungicide benomyl [6]), both *S*-alkyl *N*-alkylthiocarbamates, are even more active than the above compounds as *in vivo* ALDH2 inhibitors [2, 6] (Fig. 1). The most potent of these thiocarbamate inhibitors is MBT, acting directly or particularly via its sulfoxide, the ultimate bioactivation product of benomyl [6].

$Et_2NC(O)SMe$ sulfoxide inhibits yALDH, both the cytoplasmic and mitochondrial isoforms, by forming a carbamoylated protein identified by electrospray ionization mass spectrometry [7]. Diethylcarbamoylation also was observed with recombinant hALDH2, defining the position by HPLC-tandem mass spectrometry as Cys^{302} at the highly conserved active site region [8]. These studies used high levels of this moderately potent inhibitor, 300 and 40 μM for yALDH and hALDH2, respectively, and purified or expressed enzyme, thereby providing structural evidence for the carbamoylation reaction but not for selectivity in carbamoylating this site versus others in mitochondria.

This study tests the hypothesis that the high potency of bioactivated MBT is due to efficient sulfoxidation [6] and selective carbamoylation of mALDH2 *in situ* in liver mitochondria using bioactivated EPTC as a comparison compound. The thiocarbamates were ^{14}C-labeled at the potential carbamoylating moiety, and then subjected at low levels to *in vitro* and *in vivo* microsomal (P450) metabolism; the carbamoylated mitochondrial proteins were examined by SDS–PAGE and IEF with determination of ^{14}C-derivatized proteins by autoradiography and ALDH activity by in-gel assay. We report here that *S*-alkyl *N*-alkylthiocarbamate sulfoxides and *S*-alkyl *N*,*N*-dialkylthiocarbamate sulfoxides, exemplified by bioactivated EPTC and MBT, respectively, differed not only in potency but also in selectivity for carbamoylation of mitochondrial proteins.

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† *Abbreviations*: ALDH2, low-K_m liver mitochondrial aldehyde dehydrogenase; [^{14}C]EPTC sulfoxide, *S*-ethyl *N*,*N*-di[^{14}C]propylthiocarbamate sulfoxide; hALDH2, human ALDH2; IEF, isoelectric focusing; LSC, liquid scintillation counting; mALDH2, mouse ALDH2; MBT, *S*-methyl *N*-butylthiocarbamate; [^{14}C]MBT, *S*-methyl *N*-[^{14}C]butylthiocarbamate; MMPP, magnesium monoperoxyphthalate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; and yALDH, yeast ALDH.

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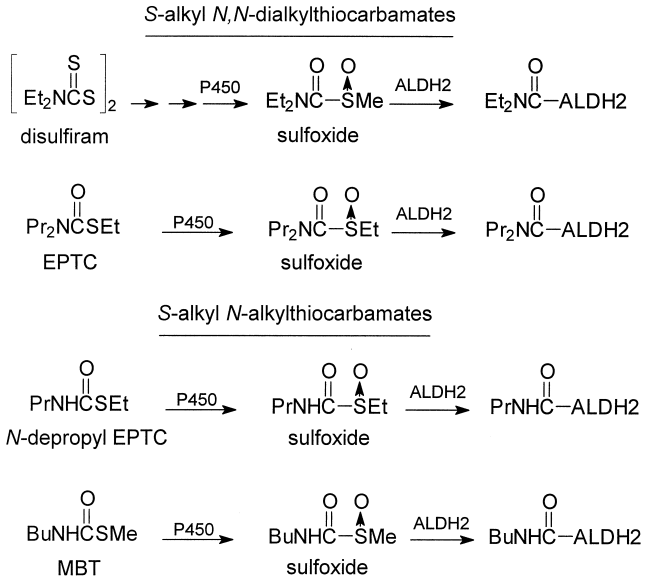

FIG. 1. Reactions involved in bioactivation of *S*-alkyl *N,N*-dialkylthiocarbamates and *S*-alkyl *N*-alkylthiocarbamates to their sulfoxides and inhibition of mALDH activity possibly by carbamoylation at Cys^{302}.

MATERIALS AND METHODS

Chemicals

[^{14}C]MBT (55 mCi/mmol, radiopurity > 96%) was synthesized previously [6], and [^{14}C]EPTC sulfoxide (15 mCi/mmol) was donated by Zeneca Ag Products. EPTC and *N*-depropyl EPTC were available from earlier studies in this laboratory [5]. EPTC sulfoxide and $Et_2NC(O)SMe$ sulfoxide were prepared by the general procedure for oxidizing thiocarbamates with one equivalent *m*-chloroperoxybenzoic acid in chloroform [9]. Sources for other chemicals were: MMPP (80% pure) from the Aldrich Chemical Co.; MTT, phenazine methosulfate, and yALDH (20–40 units/mg protein) from the Sigma Chemical Co.; and benomyl and carbendazim from Chem Service.

Chromatography, Electrophoresis, and IEF

Reversed-phase HPLC was used to examine labeled proteins with a Vydac C_4 protein column (Separations Group) (5 μm, 0.46 × 25 cm) developed with acetonitrile in water at constant 0.1% trifluoroacetic acid, initially at 0% acetonitrile for 5 min followed by linear gradients of 0–40% acetonitrile over 15 min, 40–60% over 10 min, and then 60% for 10 min, all at 1.5 mL/min. The eluent was monitored at 220 nm, and 1-min fractions were taken for LSC.

SDS–PAGE was performed on a Bio-Rad Mini-PROTEAN II system with 15 mA for 3–6 hr using 100 mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.3 as the running buffer. Separations were done on 10% Tris hand-cast gels (3.3% cross-linker) below a 5% stacking gel. Radiolabeled samples were mixed with a half-volume of Tricine® loading buffer (Bio-Rad) and heated to 90–100° for 5–7 min prior to analysis. 2-Mercaptoethanol was omitted from the loading buffer, except when specified, to avoid possible transcarbamoylation to this thiol. Proteins were stained with Coomassie blue (2.5% in 45% aqueous methanol with 10% acetic acid; 10–20 min), destained, then the gels were dried, and labeled proteins (*ca.* 500-5000 dpm) were located by autoradiography (*ca.* 2–3 days) with a Storm 860 PhosphorImager® (Molecular Dynamics).

IEF was carried out as outlined by Bio-Rad for the Mini-PROTEAN II system using precast gels (pH 5–8 Ready-Gel, Bio-Rad) with 100 V for 1 hr, 250 V for 1 hr, and finally 500 V for 30 min. The cathode buffer was substituted with Tris (32 mM) and *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) (EPPS, 30 mM) (pH 8.1) [10], while the anode buffer remained phosphoric acid (7 mM). Gels were stained with 0.05% crocein scarlet and 0.04% Coomassie blue (in 27% isopropanol, 10% acetic acid; 20–30 min), and labeled proteins were detected as above after destaining and drying the gels. Alternatively, ALDH activity was detected in IEF gels as purple bands following incubation (15–30 min in the dark) in 50 mM Tris (pH 8.5, 50 mL) containing 25 mg NAD, 2 mg phenazine methosulfate, 6 mg MTT, and 40 μL acetaldehyde [11]. The reaction was stopped with 5% acetic acid (5 min), and then the gel was rinsed with water for 30–60 min prior to drying for detection of labeled proteins as above.

Protein Labeling

yALDH (4 units, ~0.2 mg total protein) was labeled by incubation (37°, 10 min) with [^{14}C]MBT (244,000 dpm, 2 μM), mouse liver microsomes (1–2 mg), and NADPH (1 mg) in 100 mM phosphate buffer (pH 7.4, 1 mL). In competition studies, yALDH was pretreated for 10 min with unlabeled inhibitors [benomyl (~18 μM), EPTC sulfoxide (~450 μM), and $Et_2NC(O)SMe$ sulfoxide (~300 μM)] (>75% inhibition of ALDH activity [6]) prior to treatment with [^{14}C]MBT activated with MMPP [6] instead of with microsomes and NADPH.

Mouse liver mitochondria were isolated as described previously [2, 12]. Mitochondrial proteins (2.5 to 5 mg) were labeled with [^{14}C]MBT by the same method used for yALDH with microsomal/NADPH activation except that the concentration of [^{14}C]MBT was 1.5 μM. [^{14}C]EPTC sulfoxide (325,000 dpm, ~20 μM) was also used for labeling (without bioactivation). Isolated labeled mitochondria were disrupted by resuspension in distilled water and freezing; then after centrifugation (20,000 *g*, 10 min) the supernatant was analyzed by SDS–PAGE, IEF, or HPLC with LSC. Recovery of label in the soluble fraction following centrifugation averaged 83 ± 17% (SD, N = 29).

Competition studies between unlabeled inhibitors (pretreatment) and labeled inhibitors were carried out *in vitro*, *ex vivo*, and *in vivo* to determine compounds possibly acting at the same site using doses or concentrations known from previous studies to give low or high inhibition of ALDH2 activity. In the *in vitro* and *ex vivo* experiments, ALDH

activity was measured and was found to be inhibited > 75% by the unlabeled compounds prior to treatment with the radiolabel [2, 12]. Specifically, in *in vitro* competition studies, mitochondria, prior to treatment with [^{14}C]MBT as above, were pretreated for 10 min with the unlabeled inhibitors benomyl (9 μM), carbendazim (500 μM), $Et_2NC(O)SMe$ (34 μM), or MBT (3 μM) (the latter two with oxidative bioactivation; control reactions were without NADPH or microsomes). After pretreatment, the mitochondria were reisolated prior to adding [^{14}C]MBT, fresh microsomes, and NADPH. To examine *ex vivo* competition, mice were treated (i.p.) with unlabeled *N*-depropyl EPTC (22 mg/kg), EPTC (66 mg/kg), $Et_2NC(O)SMe$ (74 mg/kg), or MBT (15 mg/kg); after 2 hr the liver mitochondria were isolated [2, 12] for labeling with bioactivated [^{14}C]MBT or directly with [^{14}C]EPTC sulfoxide as above. For *in vivo* labeling, mice were treated i.p. with [^{14}C]MBT (0.01 mmol/kg, 27 mCi/mmol), and after 2 hr the mitochondria were isolated for analysis as above. In the *in vivo* competition experiments, mice were pretreated with unlabeled inhibitors [disulfiram (300 mg/kg), MBT (1.5 mg/kg), $Et_2NC(O)SMe$ (40 mg/kg), benomyl (78 mg/kg), carbendazim (52 mg/kg), or dimethyl sulfoxide (40 μL) as a control] 2 hr prior to treatment with [^{14}C]MBT (1.5 mg/kg, 27.5 mCi/mmol).

Isolation of Principal Mitochondrial Protein Labeled In Vivo *with [^{14}C]MBT for Sequence Determination*

Labeled mitochondrial proteins from mice treated with [^{14}C]MBT were purified by IEF and then recovered for SDS–PAGE in a procedure based on the two-dimensional gel separation of mALDH2 from mice treated with acetaminophen [13]. Specifically, protein from disrupted mitochondria (4 mg in 400 μL of distilled water) isolated from mice treated i.p. with [^{14}C]MBT was mixed with 100 μL IEF buffer (Bio-Rad) and separated by IEF (Bio-Rad Ready Gel, pH 5–8). As a marker, untreated mitochondrial protein (50 μg in 10 μL of distilled water mixed 1:1 with IEF buffer) was loaded in the side lane of the IEF gel adjacent to the 450-μL preparative well. After IEF, the gel was cut between the two sample wells, and the marker lane was stained for in-gel ALDH activity while the preparative lane was soaked in 50 mM Tris buffer (pH 8.5). After fixing the ALDH stain with acetic acid, the two parts of the gel were realigned, and a horizontal band was cut through the preparative lane in the region adjacent to that stained for ALDH activity (pI 6.1). The excised IEF-gel band was incubated overnight in 200 μL of Tricine sample buffer (Bio-Rad), 100 μL of 10% SDS, and 25 μL of 1.5 M Tris buffer (pH 8.5), and then heated to 90–100° for 5–10 min prior to loading onto an SDS–PAGE gel (preparative comb) for 5–6 hr development. After staining with Coomassie blue, the gel was sliced to recover the labeled protein (detected in a separate experiment by autoradiography), which was transferred to the Protein Structure Laboratory (University of California) for in-gel digestion with trypsin following reduction and derivatization with iodoacetamide. Tryptic peptide fragments were extracted from the gel with 50% aqueous acetonitrile containing 5% formic acid and fractionated by microbore HPLC. Two peptides were sequenced by an Applied Biosystems gas phase instrument, and the sequences obtained were searched for homology using the Swiss-Prot Database.

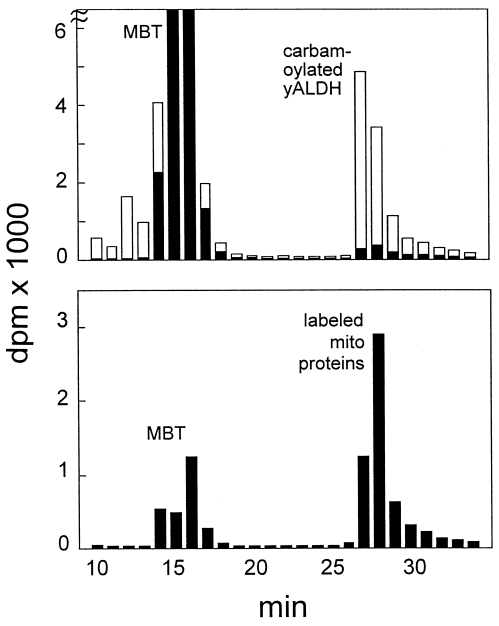

FIG. 2. Labeling *in vitro* of yALDH and mouse liver mitochondrial (mito) proteins with bioactivated [^{14}C]MBT analyzed by HPLC on a C_4 protein column. Recovery of [^{14}C]MBT after incubation with yALDH and microsomes alone (solid bars, 24,000 dpm) or microsomes plus NADPH (open bars, 10,200 dpm) is shown. For liver mitochondrial proteins, only NADPH-dependent labeling is shown (solid bars).

RESULTS

Labeling In Vitro *of yALDH with Bioactivated [^{14}C]MBT*

Initial studies considered [^{14}C]MBT incubated with yALDH and microsomes alone or microsomes plus NADPH, analyzing the products by HPLC and LSC (Fig. 2). The recovery of [^{14}C]MBT at 15–16 min was 2.4-fold greater with microsomes alone versus the same reaction including NADPH, consistent with NADPH-dependent P450 metabolism [6]. A second labeled peak eluted at 27–29 min, corresponding directly to the UV peak at 220 nm for yALDH (data not shown), with ~9000 and ~1000 dpm in the presence and absence of NADPH, respectively, as expected for ALDH labeling by P450-activated MBT. SDS–PAGE analysis with the microsome-NADPH system revealed two labeled protein bands at ~56 and 58 kDa (Fig. 3), probably the cytosolic and mitochondrial ALDHs, respectively [7]. Competitive inhibition studies established that benomyl (probably via butyl isocyanate) and the sulfoxides of EPTC and $Et_2NC(O)SMe$ blocked this label-

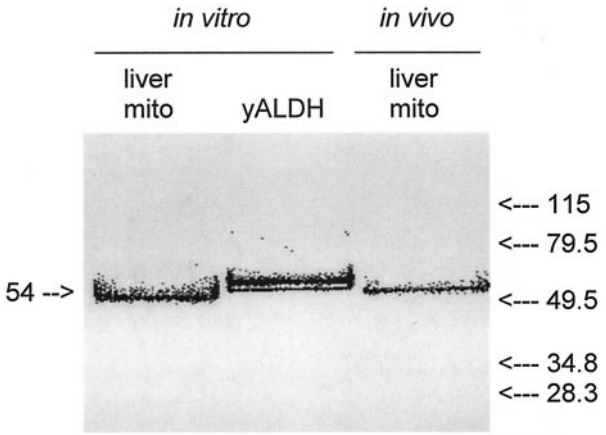

FIG. 3. Labeling of yALDH and mouse liver mitochondrial (mito) proteins with bioactivated [^{14}C]MBT analyzed by SDS–PAGE and autoradiography. For the *in vitro* studies, yALDH or mitochondria were incubated with [^{14}C]MBT, microsomes, and NADPH. For the *in vivo* study, liver mitochondria were from a mouse treated with [^{14}C]MBT. Molecular weight standards are designated on the right, and the position of the 54-kDa band is shown on the left.

ing and, therefore, derivatized the same site (data not shown).

Labeling In Vitro *and* In Vivo *of Mitochondrial Proteins with Bioactivated [^{14}C]MBT*

Mitochondrial protein(s) labeled with bioactivated [^{14}C]MBT had the same HPLC retention time as carbamoylated yALDH (Fig. 2), and appeared as predominantly one band of 54 ± 1 kDa (SD, N = 5) on SDS–PAGE (Fig. 3); a second protein sometimes was labeled weakly at ~44 kDa. The 54-kDa labeling was completely dependent upon NADPH but was similar in degree with mitochondria alone and mitochondria plus microsomes (data not shown), indicating that the bioactivation system is in mitochondria as well as microsomes [6].

Properties of Mitochondrial Proteins Labeled In Vitro *with [^{14}C]MBT*

The labeled mitochondrial preparations were also examined by IEF revealing one major (pI ~6.1) and two minor (pI ~5.8 and 5.9) labeled proteins (Fig. 4). The pI 5.9 and 6.1 labeled bands cochromatographed with the in-gel ALDH activity. These findings are consistent with bioactivated MBT *in vitro* and *in vivo* labeling predominantly ALDH2 (theoretical calculated pI 6.05; Swiss-Prot Expasy database).

Identification of Principal Mitochondrial Protein Labeled In Vivo *with [^{14}C]MBT as mALDH2*

One protein was labeled predominantly in the mitochondria of mice treated i.p. with [^{14}C]MBT, and it had the same molecular mass and pI as the mitochondrial protein labeled *in vitro* (Figs. 3 and 4). Isolation of the principal *in vivo* labeled protein region by IEF, then SDS–PAGE followed by tryptic digestion, gave two peptides with sequences matching mALDH2: (a) Ile Leu Gly Tyr Ile Lys (identical to amino acids 353 to 358), and (b) His Glu Pro Val Gly Val Cys Gly Gln Ile Ile Pro Trp Asn Phe Pro Leu (identical to amino acids 156 to 172) [14]. It should be noted that Cys and Trp are not detected by the sequencing method used. The principal labeled protein in liver mitochondria of mice treated with [^{14}C]MBT is, therefore, mALDH2.

Specificity of Unlabeled Thiocarbamates and Related Compounds as Inhibitors of Labeling of mALDH2 with Bioactivated [^{14}C]MBT

The *in vitro* and *in vivo* labeling experiments above provide a procedure to determine if unlabeled thiocarbamates and related compounds (pretreatment) compete for the same site, i.e. block the labeling of mALDH2. In studies *in vitro* with bioactivation, MBT and benomyl reduced labeling of the 54-kDa protein, but $Et_2NC(O)SMe$ and carbendazim did not (Fig. 5A); the 44-kDa protein often also underwent some labeling. Mice treated i.p. with unlabeled MBT or *N*-depropyl EPTC prior to isolation of mitochondrial proteins and treatment with [^{14}C]MBT (with microsomal/NADPH activation) had reduced labeling of mALDH2 compared with untreated control mice, but similar treatments with EPTC and $Et_2NC(O)SMe$ did not inhibit this *ex vivo* labeling (Fig. 5B). These same relationships were applicable to *in vivo* studies. Thus, pretreating mice with unlabeled MBT or benomyl prior to i.p. treatment with [^{14}C]MBT decreased labeling of mALDH2, whereas pretreatment with carbendazim, disulfiram, or $Et_2NC(O)SMe$ did not (Fig. 5, C and D).

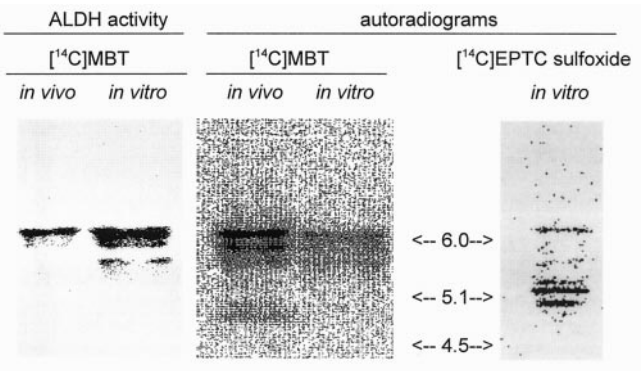

FIG. 4. Liver mitochondrial proteins labeled *in vivo* and *in vitro* with bioactivated [^{14}C]MBT and *in vitro* with [^{14}C]EPTC sulfoxide analyzed by IEF with in-gel ALDH staining and/or autoradiography. The ALDH and autoradiographic assays for [^{14}C]MBT (four left lanes) are cochromatograms of the same gel. pI standards are designated.

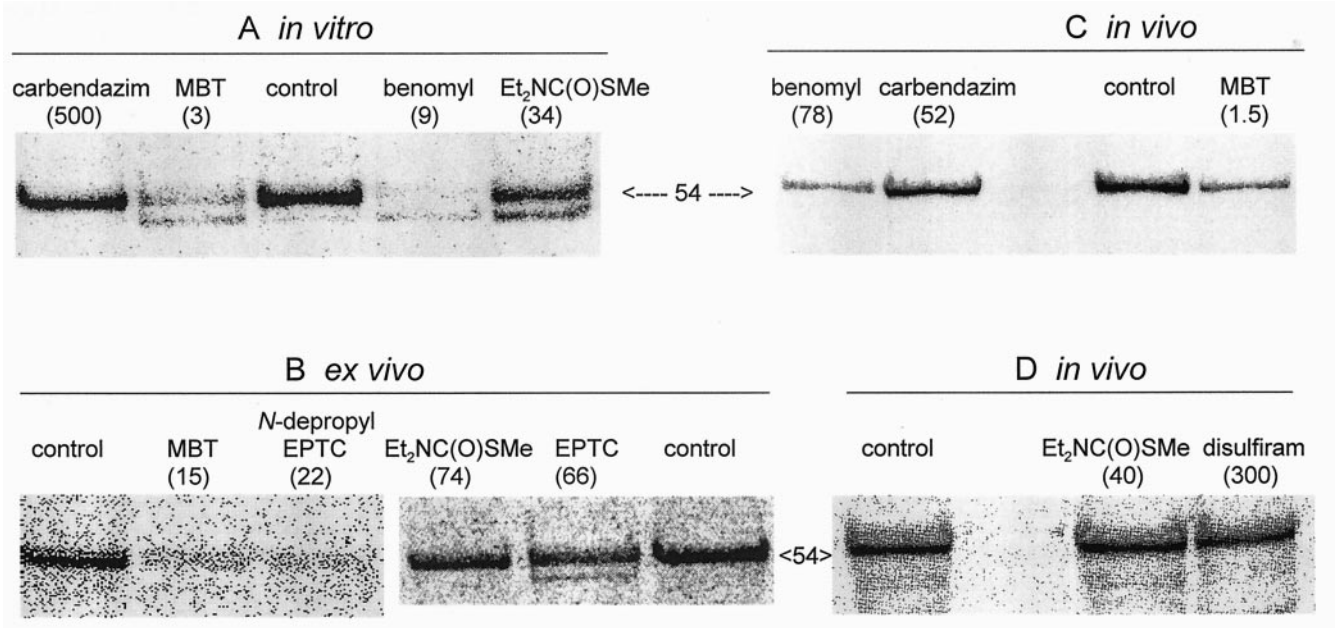

FIG. 5. Specificity of unlabeled thiocarbamates and related compounds as inhibitors of labeling of liver mALDH2 *in vitro*, *ex vivo*, and *in vivo* with bioactivated [^{14}C]MBT, analyzed by SDS–PAGE and autoradiography. Pretreatments with unlabeled compounds are stated (in parentheses) as micromolar concentrations *in vitro* and milligram per kilogram doses *ex vivo* and *in vivo*. Each gel is a separate experiment. The position of the 54-kDa band is designated.

Labeling In Vitro *and* In Vivo *of Mitochondrial Proteins with [^{14}C]EPTC Sulfoxide Compared with Bioactivated [^{14}C]MBT*

LABELING *IN VITRO* WITH [^{14}C]EPTC SULFOXIDE AND BIOACTIVATED [^{14}C]MBT. Incubation of mitochondria with [^{14}C]EPTC sulfoxide resulted in predominant labeling of two proteins at 58 ± 3 and 61 ± 3 kDa (SD, N = 3) (Figs. 6 and 7). A protein at ~44 kDa was labeled to a lesser extent. IEF separated four labeled proteins with approximate pI values of 6.2, 5.3, 5.2, and 5.0 (Fig. 4), with the pI 5.2 band labeled the most. Only the minor pI 6.2 band was in the region of ALDH activity detected by in-gel analysis (data not shown). The primary proteins labeled by [^{14}C]EPTC sulfoxide did not include mALDH2 based on comparison with labeling by bioactivated [^{14}C]MBT when analyzed as individual and mixed samples (Fig. 6).

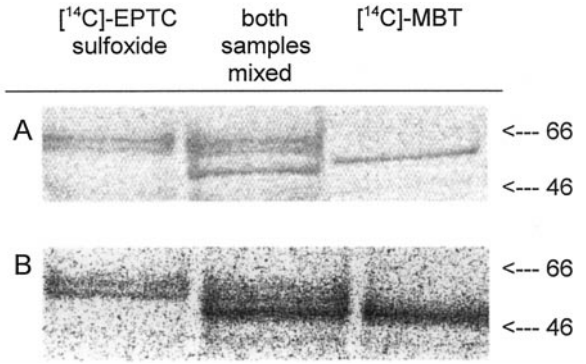

FIG. 6. Labeling *in vitro* of mouse liver mitochondrial proteins with [^{14}C]EPTC sulfoxide (20 μM) and bioactivated [^{14}C]MBT (1.5 μM) analyzed by SDS–PAGE and autoradiography. Samples for gel A but not for gel B were denatured in the presence of 2% 2-mercaptoethanol, leading to better resolution but loss of some label. Molecular weight standards are designated.

Specificity of Unlabeled Thiocarbamates as Ex Vivo *Inhibitors of Labeling of Mitochondrial Proteins with [^{14}C]EPTC Sulfoxide*

Mice treated i.p. with unlabeled MBT or *N*-depropyl EPTC prior to isolation of mitochondrial proteins and treatment with [^{14}C]EPTC sulfoxide had labeling similar to that of control mice (Fig. 7). In contrast, pretreatment with EPTC or $Et_2NC(O)SMe$ completely blocked subsequent labeling by [^{14}C]EPTC sulfoxide (Fig. 7).

DISCUSSION

MBT, via its metabolite MBT sulfoxide, is the most potent thiocarbamate reported to date as an inhibitor of mALDH2, with an *in vivo* IC_{50} for MBT of 0.32 mg/kg and

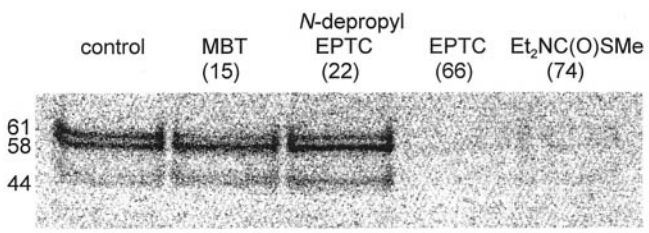

FIG. 7. Specificity of unlabeled thiocarbamates as *ex vivo* inhibitors of labeling of liver mitochondrial proteins with [^{14}C]EPTC sulfoxide. Pretreatment with unlabeled compounds was at the milligram per kilogram doses indicated (in parentheses). Positions of the 44-, 58-, and 61-kDa bands are designated.

an *in vitro* IC_{50} for MBT sulfoxide of 0.089 μM [6]. In comparison, the sulfoxides of EPTC and $Et_2NC(O)SMe$ are at least 8-fold (in rats [3]) and 12-fold (in mice [2]) less potent both *in vivo* and *in vitro*. The present study compared bioactivated MBT with EPTC sulfoxide relative to their reactions with mouse mitochondrial proteins.

Discovery of the high potency of MBT sulfoxide arose from metabolic studies on the fungicide benomyl [6] rather than from optimization of *S*-alkyl *N*-alkylthiocarbamates and their sulfoxides for maximum effectiveness. The biological activity of thiocarbamates is highly dependent on the balance of *S*-alkyl and *N*-alkyl substituents in assays as herbicides [15] and nematicides [16] and probably as ALDH2 inhibitors. The direct approach of a structure–activity study on *S*-alkyl *N*-alkylthiocarbamate sulfoxides from synthesis is not easily achieved because of the need for pure and characterized sulfoxides, which are only accessible for now by aqueous peracid oxidation of the thiocarbamates, and once obtained they are unstable, e.g. half-life of 6 min for MBT sulfoxide at pH 7.4 and 22° [6].

The mALDH2 site at which MBT sulfoxide reacts is presumably Cys^{302}, based on analogy with the reaction of $Et_2NC(O)SMe$ sulfoxide with recombinant hALDH2 [8], and the critical importance of this residue for enzyme activity [17, 18] (Fig. 1). Characterization of the carbamoylated site is more difficult with *N*-alkylcarbamoyl than *N,N*-dialkylcarbamoyl proteins because of the greater lability of the *N*-monoalkyl derivatives, and, therefore, the isolation and sequencing methods must be under near-neutral conditions with minimal exposure to thiols. A suitable peptide adduct for sequencing was recovered for hALDH2 carbamoylated with $Et_2NC(O)SMe$ sulfoxide by digestion at pH 3.7 with endopeptidase Glu-C but not with trypsin at pH 8.5 [8]. In the present study, both of these proteolytic conditions hydrolyzed the *N*-butylcarbamoyl adduct of mALDH2.

The principal mitochondrial protein derivatized in the livers of mice by bioactivated MBT *in vitro* and *in vivo* was mALDH2, and *N*-depropyl EPTC appeared to have the same specificity, which therefore may be conferred by *S*-alkyl *N*-alkylthiocarbamate sulfoxides. In contrast, the major mitochondrial proteins labeled by [^{14}C]EPTC sulfoxide *in vitro* differed from those labeled by [^{14}C]MBT. Further, disulfiram, its metabolite $Et_2NC(O)SMe$, and EPTC, following bioactivation, did not block labeling by [^{14}C]MBT, suggesting that there is both a potency and a specificity difference between the sulfoxides of the *S*-alkyl *N*-alkylthiocarbamates and those of the S-alkyl *N,N*-dialkylthiocarbamates. The ability of *N*-depropyl EPTC, but not EPTC, to block labeling of mALDH2 by [^{14}C]MBT in *ex vivo* experiments demonstrates that *N*-dealkylation of EPTC is a minor metabolic pathway [5]. The principal labeled mitochondrial proteins from EPTC sulfoxide are probably not ALDHs, based on their pI values and in-gel ALDH assays, but one of them (~61 kDa) is similar in molecular mass to liver microsomal hydrolase A inhibited by molinate sulfoxide (another thiocarbamate sulfoxide) [19].

This investigation considered bioactivation of thiocarbamates to sulfoxides as inhibitors of ALDH2. MBT sulfoxide is, at present, the most potent and selective carbamoylating agent for the active site of mALDH2. These results are relevant to alcohol aversion upon exposure to herbicides, fungicides, and other inhibitors of mALDH2.

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