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Biochemical Pharmacology 61 (2001) 537–545

Biochemical Pharmacology

Determination of *in vivo* adducts of disulfiram with mitochondrial aldehyde dehydrogenase

Maryann L. Shen[a,b], Kenneth L. Johnson[b,c], Dennis C. Mays[a], James J. Lipsky[a,d,e], Stephen Naylor[a,b,c,d,*]

[a]*Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic/Foundation, 200 First Street SW, Rochester, MN 55905, USA*
[b]*Biomedical Mass Spectrometry and Functional Proteomics Facility, Guggenheim C-009B, 200 First Street SW, Mayo Clinic/Foundation, Rochester, MN 55905, USA*
[c]*Department of Biochemistry and Molecular Biology, Mayo Clinic/Foundation, 200 First Street SW, Rochester, MN 55905, USA*
[d]*Clinical Pharmacology Unit, Mayo Clinic/Foundation, 200 First Street SW, Rochester, MN 55905, USA*
[e]*Department of Internal Medicine, Mayo Clinic/Foundation, 200 First Street SW, Rochester, MN 55905, USA*

Received 17 May 2000; accepted 21 July 2000

Abstract

Extensive use for disulfiram (DSF) has been found in the aversion therapy treatment of recovering alcoholics. Although it is known to irreversibly inhibit hepatic aldehyde dehydrogenase (ALDH), the specific mechanism of *in vivo* inhibition of the enzyme by the drug has not been determined yet. We have demonstrated in this report a novel, but simple and rapid method for structurally characterizing *in vivo* derived protein–drug adducts by linking on-line sample processing to HPLC–electrospray ionization mass spectrometry (HPLC–MS) and HPLC–tandem mass spectrometry (HPLC–MS/MS). Employing this approach, rats were administered DSF, and their liver mitochondria were isolated and solubilized. Both native and *in vivo* DSF-treated mitochondrial ALDH (mALDH) were purified in one step with an affinity cartridge. The *in vivo* DSF-treated mALDH showed 77% inhibition in enzyme activity as compared with that of the control. Subsequently, the control and DSF-inhibited mALDH were both subjected to HPLC–MS analyses. We were able to detect two adducts on DSF-inhibited mALDH, as indicated by the mass increases of ~71 and ~100 Da. To unequivocally determine the site and structure of these adducts, on-line pepsin digestion-HPLC–MS and HPLC–MS/MS were performed. We observed two new peptides at $MH^+ = 973.7$ and $MH^+ = 1001.8$ in the pepsin digestion of DSF-inhibited enzyme. These two peptides were subsequently subjected to HPLC–MS/MS for sequence determination. Both peptides possessed the sequence $FNQGQC_{301}C_{302}C_{303}$, derived from the enzyme active site region, and were modified at Cys_{302} by *N*-ethylcarbamoyl (+71 Da) and *N*-diethylcarbamoyl (+99 Da) adducts. These findings indicated that *N*-dealkylation may be an important step in DSF metabolism, and that the inhibition of ALDH occurred by carbamoylation caused by one of the DSF metabolites, most likely *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide (MeDTC-SO). Finally, there was no evidence of the presence of an intramolecule disulfide bridge modification on the peptide FNQGQCCC.

Keywords: Aldehyde dehydrogenase; Disulfiram; *In vivo* enzyme inhibition; Mass spectrometry; Protein–drug interactions

1. Introduction

Antabuse (bis[diethylthiocarbamoyl] disulfide), also known as DSF, has been widely used clinically in the aversion therapy treatment of recovering alcoholics for over 50 years [1]. It is believed to be an irreversible inhibitor of mALDH, one of the key enzymes involved in alcohol metabolism [2]. Inhibition of ALDH leads to the accumulation of acetaldehyde after imbibing ethanol, and, thereby, induces a distinctly unpleasant DSF–ethanol reaction characterized by vasodilation, tachypnea, and tachycardia with subsequent nausea, vomiting, and hypotension [1]. DSF, the

* Corresponding author. Tel.: +1-507-284-5220; fax: +1-507-284-8433.
E-mail address: naylor.stephen@mayo.edu (S. Naylor).

Abbreviations: DSF, disulfiram; mALDH, hepatic mitochondrial aldehyde dehydrogenase; DDC, diethyldithiocarbamate; HPLC–MS, on-line HPLC–electrospray ionization mass spectrometry; HPLC–MS/MS, on-line HPLC–tandem mass spectrometry; MeDTC-SO, *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide; MeDTC-SO_2, *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfone; MeMETC-SO, *S*-methyl-*N*-monoethylthiocarbamoyl sulfoxide; ESI–MS, electrospray ionization–mass spectrometry; and MS/MS, tandem mass spectrometry.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00586-4

parent drug, is rapidly reduced *in vivo* to DDC [3]; however, it has been proposed that reoxidation of DDC to DSF is responsible for actual ALDH inhibition [4–10]. It has also been suggested that metabolites, such as MeDTC-SO and MeDTC-SO_2, are responsible for the *in vivo* inactivation of the enzyme [11–13]. Previous studies have shown that MeDTC-SO is a potent, irreversible inhibitor of ALDH *in vitro* and *in vivo* [11,13,14]. In addition, this metabolite has been detected in rat plasma after DSF administration [11, 15,16]. MeDTC-SO also inhibits recombinant human mALDH *in vitro* by forming a covalent adduct on one of the essential cysteines at the active site of the enzyme [17]. MeDTC-SO_2 is also a potent inhibitor of ALDH *in vitro* [13,14], yet, possibly due to its reactive nature, it has never been detected *in vivo.* In spite of the widespread use of this drug, the mechanism of inhibition of ALDH by DSF *in vivo* has not been determined to date.

Numerous strategies have been reported that purport to describe the interaction of a drug with its target protein(s). Such approaches typically include enzyme activity/inhibition assays, immunofluorescence, radioactive labeling, cross-linking, and protein binding assays, which all utilize nonspecific detection systems and in some cases require significant amounts of protein [18]. Furthermore, this information does not allow facile identification of the target protein, or determination of the mechanism of inhibition by the drug or its metabolite. More recently, electrospray ionization (or miniaturized versions such as microelectrospray and nanospray) in conjunction with mass spectrometry (ESI–MS) has played an increasingly important role in the rapid identification of proteins [19]. In particular, ESI–MS is now one of the preferred methods of choice in proteomic [20], functional proteomic [21], and functional genomic [22,23] analyses. Furthermore, ESI–MS is proving to be highly complementary to multidimensional NMR. The former allows rapid structural determination of both native and modified proteins, requiring only femtomole–attomole amounts of analyte, while affording primary sequence information [19,20].

In the present work, we describe a new, simple, but rapid, on-line sample processing and chromatographic separation method, used in conjunction with ESI–MS and ESI–tandem-MS (ESI–MS/MS) to structurally characterize *in vivo* derived protein–drug adducts, that overcomes the limitations of other generally used techniques (shown schematically in Fig. 1). We demonstrate the power of such an approach in determining both the site of adduct formation and the structure of the DSF metabolite–mALDH adduct produced after administering DSF to rats.

2. Materials and methods

2.1. ALDH activity assay

The enzyme activity assay was performed as previously described [24], with some minor modifications. Briefly, 10 μL of purified mALDH was dispensed into the microtiter plate well in triplicates followed by the addition of 190 μL of 50 mM sodium pyrophosphate buffer, pH 8.8, and 25 μL of acetaldehyde and NAD^+. The final acetaldehyde concentration was 160 μM, and that of NAD^+ was 500 μM. Enzyme activity was determined, in part, by spectrophotometrically monitoring NADH formation at 340 nm using a THERMOmax Microplate Reader (Molecular Devices).

2.2. Purification of mALDH

Rats were administered DSF i.p. at 75 mg/kg in 0.5% methylcellulose after fasting for 16 hr. Following the injection, rats were fasted for another 18 hr and killed. Rat liver was homogenized, and mitochondria were isolated by differential centrifugation as described [25]. Mitochondria were solubilized with 0.25 mg deoxycholate/mg protein, and the debris was removed by centrifugation at 150,000 *g* for 45 min. The supernatant was subsequently passed through an α-cyano-4-hydroxycinnamate affinity cartridge. The coupling of α-cyano-4-hydroxycinnamate to the Sepharose CL-4B solid support has been described in detail elsewhere [26]. The soluble mitochondrial protein mixture was applied to the affinity cartridge already equilibrated in 50 mM NaCl/2 mM benzamidine/1 mM EDTA/0.1 mM dithiothreitol/20 mM MOPS, neutralized to pH 7.4 with NaOH. The cartridge was washed free of unbound proteins with equilibration buffer described above, and subsequently mALDH was eluted in buffer containing 2 mM α-cyano-4-hydroxycinnamate. Protein concentration of the eluant fraction was measured by BCA Kinetic Protein Assay using bovine serum albumin as the standard. Fractions with protein concentration of $>$0.5 mg/mL were collected.

2.3. On-line pepsin digestion

Pepsin digestions of both control and *in vivo* DSF-inhibited ALDH were performed on-line by linking a digestion cartridge made by packing a microbore guard column of 1×20 mm (Upchurch Scientific) with pepsin 1000 Å pore size Porozyme medium (PerSeptive Biosystems) with HPLC–MS. To generate the digestion product, 80 μL of mALDH was first incubated with 8 μL of 8 M urea for 5 min, which then was diluted by adding 140 μL of buffer (20 mM ammonium acetate, 1% acetic acid, pH 3.7). This mixture was infused through the cartridge at 3 μL/min at 45° with a Michrom UMA (Michrom Bioresources) injector at load position so that the effluent was loaded onto a 1×10 mm reversed-phase polymer-based peptide microtrap (Michrom Bioresources) in place of the sample loop. After infusing the enzyme solution, the Porozyme cartridge was flushed with 100 μL of ammonium acetate buffer at 5 μL/min. As the injector was switched to inject mode, the peptide microtrap was subsequently placed in-line with the LC column, and the peptides were eluted by increasing organic content.

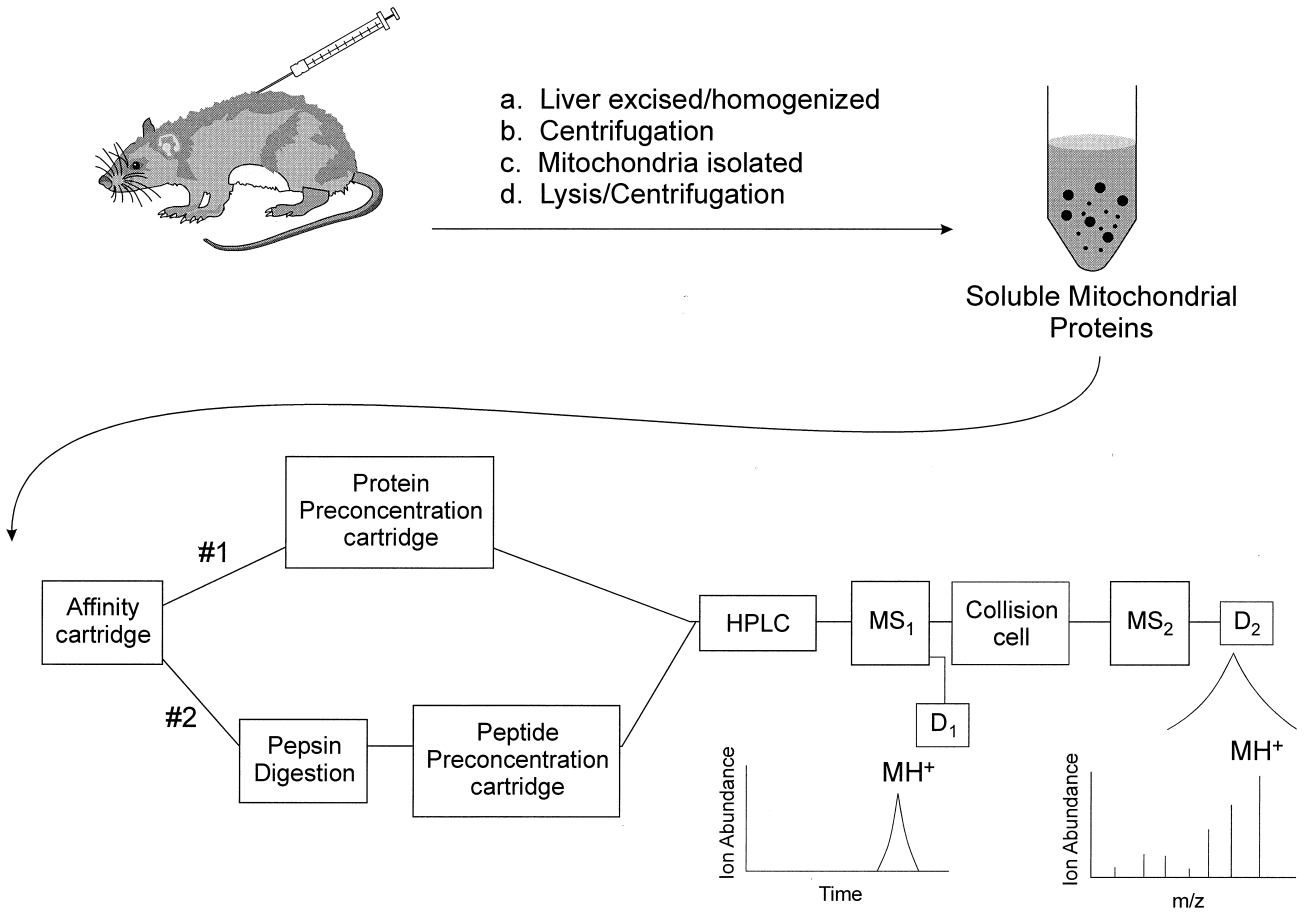

Fig. 1. General scheme for the rapid isolation and structural characterization of *in vivo*-derived DSF metabolite ALDH adduct(s). Rats were administered DSF in 0.5% methylcellulose, i.p., and killed after 18 hr. Liver mitochondria were isolated and solubilized. Rat liver mALDH was purified using an α-cyano-4-hydroxycinnamate affinity column. Along pathway 1, intact mALDH (DSF-treated or control) was preconcentrated on a C-4 protein cartridge trap, and then subjected to HPLC–MS to determine molecular masses of the protein as detected at D_1. Detailed structural information was obtained via pathway 2, employing on-line pepsin digestion HPLC–MS and HPLC–MS/MS. Specifically, mALDH was infused through a Porozyme digestion cartridge with a Michrom UMA injector at load position so that the effluent was loaded onto the reversed-phase peptide trap. After infusing the enzyme solution, the injection valve was switched to inject position, and the peptide trap was thus connected with the reversed-phase column. Peptides were eluted by increasing the organic content in the mobile phase. The entire effluent was introduced into the electrospray interface, and analyzed by HPLC–MS and HPLC–MS/MS with product ion data being acquired at D_2.

2.4. HPLC–MS and HPLC–MS/MS

Intact mALDH was analyzed by HPLC–MS using a Finnigan MAT 900 mass spectrometer (Finnigan MAT GmbH) with a Finnigan MAT designed electrospray interface. HPLC separation was carried out on Michrom UMA using a PLRP-S reversed-phase, 300 Å pore size, 1.0 x 50.0 mm column (Michrom Bioresources). Mobile phase A (98:1:1:0.5:0.02, by vol., water:acetonitrile:*n*-propanol:acetic acid:trifluoroacetic acid) and mobile phase B (80:10:10:0.5:0.02, by vol., acetonitrile:water:*n*-propanol:acetic acid:trifluoroacetic acid) were used to create a gradient of 20–70% B over 10 min at a flow rate of 75 μL/min. Intact control and inhibited mALDH (25 μL) were preconcentrated onto a 1 $\times$ 10 mm polymer-based reversed-phase protein microtrap with 4000 Å pore size (Michrom Bioresources), which replaced the sample loop of the HPLC injector. Salts and other components were removed by washing the trap with 200 μL of mobile phase A before the preconcentration trap was placed in-line with the column for development of the gradient. The entire effluent was introduced into the electrospray interface and ionized. The electrospray voltage used was 4.14 kV with N_2 as the sheath gas at 2.5 L/min. The ions generated were detected by a PATRIC (Position and Time Resolved Ion Counter) scanning array detector using an 8% mass window. HPLC separations of peptides from on-line pepsin digestion of either control or inhibited mADLH were performed using a Monitor C18 (Michrom Bioresources), 100 Å pore size, 1.0 $\times$ 150 mm column. A gradient of 2–70% mobile phase B over 30 min at a flow rate of 50 μL/min was applied. The peptides were analyzed by a Sciex API 365 mass spectrometer (Sciex) with electrospray ionization. The mass spectrometer was scanned from *m/z* of 400 to 1800 with a step of 0.1 a.m.u. at 0.1 msec/step. An ionspray voltage of 5100 V, an orifice voltage of 55 V, and a ring voltage of 350 V were used for the

analysis of the peptides. Tandem mass spectrometry was accomplished utilizing a Sciex API 365 mass spectrometer with electrospray ionization. Nitrogen was used as the CAD gas, and the CAD gas control was set at 5 with a collision energy of −41.5 V (difference between R01 and R02). The resolution of Q1 was set at 2 a.m.u. and that of Q3 at 3 a.m.u. at the base.

3. Results

DSF has been used as an aversion therapy treatment for recovering alcoholics for over five decades [1]. However, the *in vivo* mechanism of inhibition still has not been directly determined. Hence, we were interested in discovering the precise mechanisms and site of inhibition of mALDH *in vivo*.

3.1. Rapid screen to determine in vivo protein modification

Initially, we evaluated the usefulness of the α-cyano-4-hydroxycinnamate column to selectively isolate mALDH. Rats were administered 75 mg/kg of DSF i.p., and along with control rats were killed after 18 hr. After liver excision and processing, the mitochondrial fraction was subjected to affinity chromatography purification. Analysis by SDS–PAGE revealed a single band at ~55 kDa, after silver staining, in both the control and the DSF-treated rat eluate (data not shown). This demonstrated that the affinity column worked, but that either no modification of mALDH in DSF-treated rats had occurred or the SDS–PAGE had inadequate resolving capability. However, mALDH activity of both mitochondrial-derived protein and affinity-purified protein was also measured. In the DSF-treated rats, the specific activity of mALDH in solubilized mitochondria was 2.24 nmol NADH/mg protein compared with 6.34 nmol NADH/mg protein in control rats. The DSF decreased enzyme activity by ~65%. In addition, the specific activity of affinity-purified mALDH from DSF-treated rats was decreased by ~77% compared with that of the control. This latter observation is consistent with a previous report that ~75% inhibition was achieved 18 hr after DSF administration at the same dose [27].

To determine if mALDH had been modified by metabolite adduction, the DSF-treated and control mitochondrial liver protein fractions were subjected directly to affinity chromatography coupled with HPLC–MS (Fig. 1, pathway 1). The molecular mass of the intact control mALDH protein was determined to be 54,410 Da (±0.01%) (Fig. 2A), while the *in vivo* DSF-inhibited enzyme showed three different molecular masses of 54,410, 54,481, and 54,510 Da (±0.01%) (Fig. 2B). The molecular mass of 54,410 Da indicated the presence of unmodified mALDH, which could explain the remaining enzyme activity detected after treatment with DSF. The increase in molecular mass of ~71 and ~100 Da to 54,481 and 54,510 Da (Fig. 2B), respectively, suggested that DSF inhibited mALDH *in vivo* by forming two different, discrete adducts on the protein.

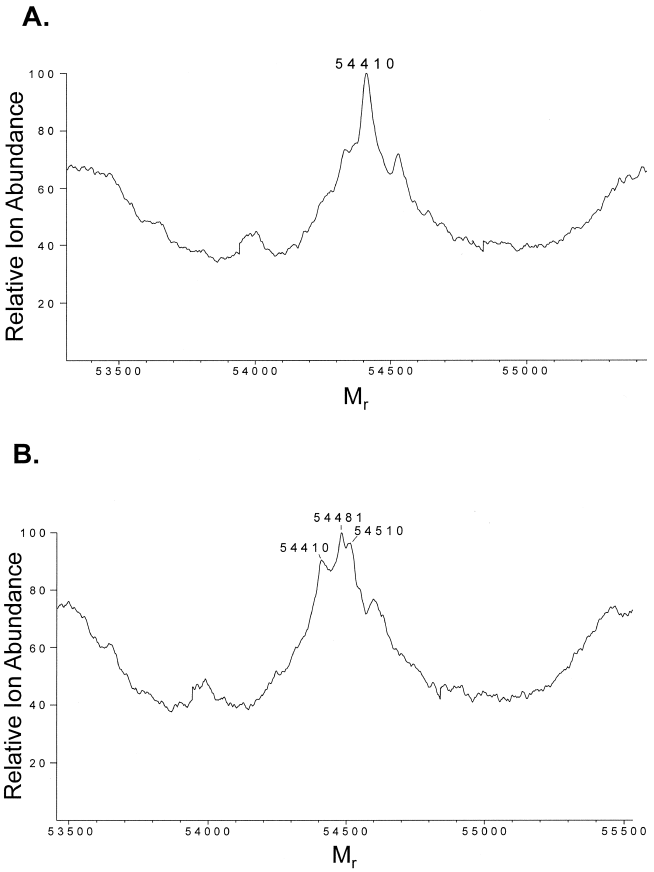

Fig. 2. HPLC–MS analysis of affinity-purified rat liver mALDH, preconcentrated prior to analysis. (A) Intact mALDH obtained from a control rat and subjected to ESI–MS analysis. The transformed raw data afforded a M_r = 54,410 (±0.01% mass error). (B) Intact mALDH obtained from a DSF-treated rat and subjected to ESI–MS. After transformation of the raw data, unmodified mALDH (M_r = 54,410) was still observed but now two new ion responses at M_r = 54,481 and 54,510 could be detected, corresponding to adducted mALDH protein with net mass increases of ~71 and ~100 Da (±0.01% mass error), respectively.

3.2. On-line pepsin digestion HPLC–MS

To determine both the site and structure of the mALDH–DSF metabolite adduct, it was necessary to proteolytically digest the protein. We have demonstrated previously '*in vitro*' that some DSF metabolite-derived adducts are base labile [17]. Hence, the pH-dependent lability of peptide adducts coupled with the molecular mass range constraints of peptides needed for efficient MS/MS sequence analysis necessitated: (a) that the proteolytic enzyme should have a V_{max} that is optimal at acidic pH; and (b) the production of peptides in the molecular mass range ~500–2000 Da. Pepsin is just such a proteolytic enzyme that cleaves at the N-terminal side of hydrophobic amino acids including Phe, Leu, Ile, Try, and Ala and has a V_{max} at pH ~4.0 [28].

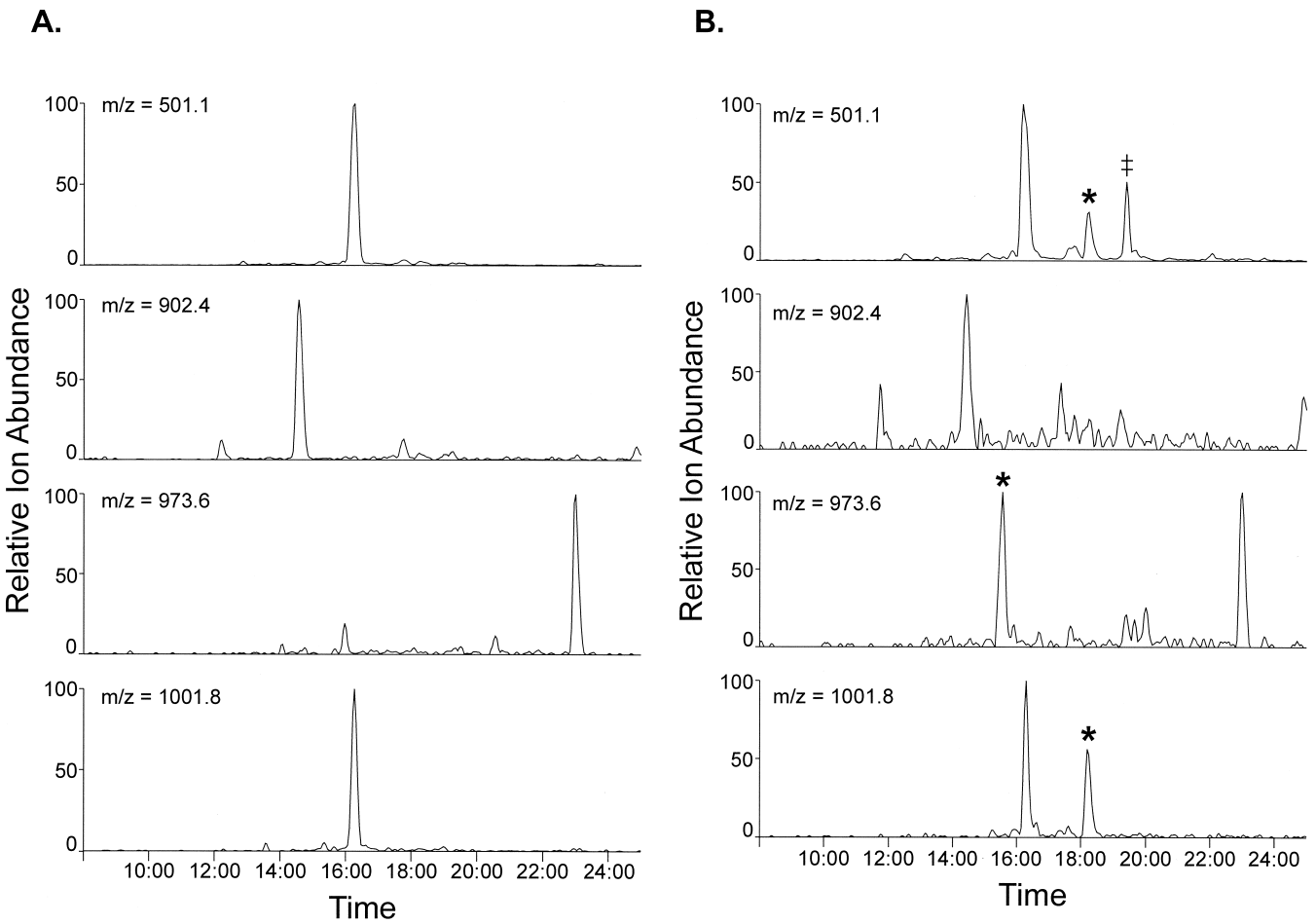

Fig. 3. Affinity-purified mALDH subsequently subjected to on-line pepsin proteolytic digestion, followed by preconcentration on a peptide cartridge trap and subsequent HPLC–MS analysis, employing ESI–MS with detection at D_1 (see pathway 2, Fig. 1). (A) Partial HPLC–MS analysis of mALDH from control rats after on-line pepsin digestion. Each ion chromatogram represents a single ion mass measured over the time of the analysis. The ion at *m/z* 1001.8 is the MH_2^{2+} species of a pepsin-derived peptide, NRLADLIERDRTYLAAL, observed previously [17]. The ion at *m/z* 501.1 is the MH_4^{4+} of the ion at *m/z* 1001.8. The ion at *m/z* 973.6 is a singly charged ion (MH^+) of a mALDH peptide, YAGWADKY, also derived from the pepsin digestion. The ion at *m/z* = 902.4 corresponds to the native peptide FNQGQCCC derived from the active site of mALDH [17]. (B) HPLC–MS analysis of mALDH from rats treated i.p. with DSF, after on-line pepsin digestion. Ions at *m/z* = 973.7, 1001.8 and MH_2^{2+} = 501.1 were observed (indicated by *), which were not present in the digest of the native protein shown in (A). These ions can be assigned to the peptide $FNQGQC_{301}C_{302}C_{303}$ of MH^+ = 902.4 with adducts of 71 Da and 99 Da, respectively. Note that the response at *m/z* 501.1 with a retention time of 20.00 min, (‡), was found transiently sometimes in both 'control' and DSF-treated samples.

Hence, the affinity-isolated 'control' and the DSF-treated mALDH were individually subjected to on-line pepsin digestion. The resulting proteolytic peptides were concentrated on the reversed-phase peptide trap and subsequently analyzed by HPLC–MS (see Fig. 1, pathway 2), and the resulting ion chromatograms were compared. The HPLC –MS of the 'control' mALDH revealed a plethora of peptide responses (data not shown). Upon careful inspection, a singly charged protonated molecular ion (MH^+ at *m/z* = 902.4) could be observed (Fig. 3A) that corresponded to the unmodified peptide FNQGQCCC. This peptide contains three adjacent cysteine residues, is found at the active site of mALDH, and has been identified previously in the HPLC–MS of recombinant human mALDH [17]. The ions detected at *m/z* 1001.8 and 501.1 (retention time 16.25 min) in the 'control' are the MH_2^{2+} and MH_4^{4+} ions of the peptide NRLADLIERDRTYLAAL, also detected previously in a pepsin digest of control recombinant human mALDH [18]. Also, the ion at *m/z* 973.6 (Fig. 3A) is the singly charged (MH^+) peptide YAGWADKY.

A pepsin digest of DSF-treated mALDH, carried out under identical conditions, revealed a similar complex pattern of peptides. The ion abundance at *m/z* = 902.4 was reduced almost 60% (Fig. 3B) compared with the HPLC –MS of the control mALDH (Fig. 3A). Furthermore, three new peptide responses (marked with * in Fig. 3B), not present in control mALDH analyses, were detected at *m/z* 501.1, 973.6, and 1001.8. It should be noted that the ion at *m/z* 501.1 with a retention time of ~20.00 min (denoted with ‡ in Fig. 3B) was transiently detected sometimes in both control and DSF-inhibited mALDH. The ions at *m/z* 973.6 could be tentatively assigned to the singly charged

peptide FNQGQCCC of MH^+ = 902.4, with a covalent adduct modification of 71 Da. The ions at *m/z* 1001.8 and 501.1 (* in Fig. 3B) can be tentatively assigned as the singly (MH^+) and doubly charged (MH_2^{2+}) ions, respectively, of the same peptide, but now with a covalent modification of 99 Da. It also should be noted that there was no evidence for the formation of an internal disulfide bridge being formed on the FNQGQCCC peptide caused by reduction of the active site by DSF or one of its metabolites.

3.3. On-line pepsin digestion HPLC–MS/MS

In many instances, the primary structure of a protein can be readily determined from either the cDNA sequence of the gene or conventional Edman chemistry. However, where chemical or post-translational modifications have occurred, the use of MS/MS has played a key role in determining the nature and site of adduction after the protein has been subjected to proteolytic digestion [29,30]. We have previously discussed optimal conditions for the collisionally induced dissociation (CID) of peptides [31]. Employing similar collision conditions, we subjected the modified peptide adducts at MH^+ = 973.7 and MH^+ = 1001.8 to HPLC–MS/MS. In the HPLC–MS/MS analyses of both singly charged species MH^+ = 973.7 (Fig. 4A) and MH^+ = 1001.8 (Fig. 4B), they each afforded a series of 'b' ions, namely b_2, b_3, b_4, b_5, and b_6 at *m/z* = 262, 390, 447, 575, and 678, respectively, as well as a corresponding series of 'b*' ions (b_2*, b_3*, b_4*, b_5*, and b_6*) at *m/z* = 245, 373, 430, 558, and 661, respectively.

The 'b' and 'b*' ion series correspond to fragmentation of the amide backbone of the peptide with charge retention at the N-terminus, and this is shown schematically in Fig. 4C (note that the nomenclature is after that of Roepstorff and Fohlman) [32]. These common b and b* product ions shown in panels A and B of Fig. 4 indicate the presence of an unmodified partial sequence FNQGQC, known to be present at the active site of the enzyme [33–36]. Careful inspection of the product ion spectrum of the 71 Da adduct peptide of MH^+ = 973.7 (Fig. 4A) revealed that the adduct was formed on the Cys_{302} residue of the peptide $FNQGQC_{301}C_{302}C_{303}$ (Fig. 5A). This was predicated on the fact that a series of 'y' ions (peptide backbone cleavage with charge-retention at the C-terminal; Fig. 4C) were observed containing the adduct, and labeled y_{2A}, y_{3A}, y_{4A}, y_{5A}, y_{6A}, y_{4A}*, y_{6A}*, and y_{8A}* detected at *m/z* = 296, 399, 527, 584, 712, 510, 695, and 956, respectively. This was definitively confirmed by the presence of adducted 'b' ions, b_{7A}, b_{8A}, and b_{7A}* at *m/z* = 852, 955, and 835, respectively. In addition, an ion at *m/z* = 902 also was observed corresponding to the MH^+ of unadducted peptide $FNQGQC_{301}C_{302}C_{303}$, confirming that the adduct adds 71 Da to the peptide. This represents the adduction of an *N*-ethylcarbamoyl product, and the structure is shown in Fig. 5A. The detection of an *N*-ethylcarbamoyl adduct on Cys_{302} is in agreement with a previous report that an *N*-

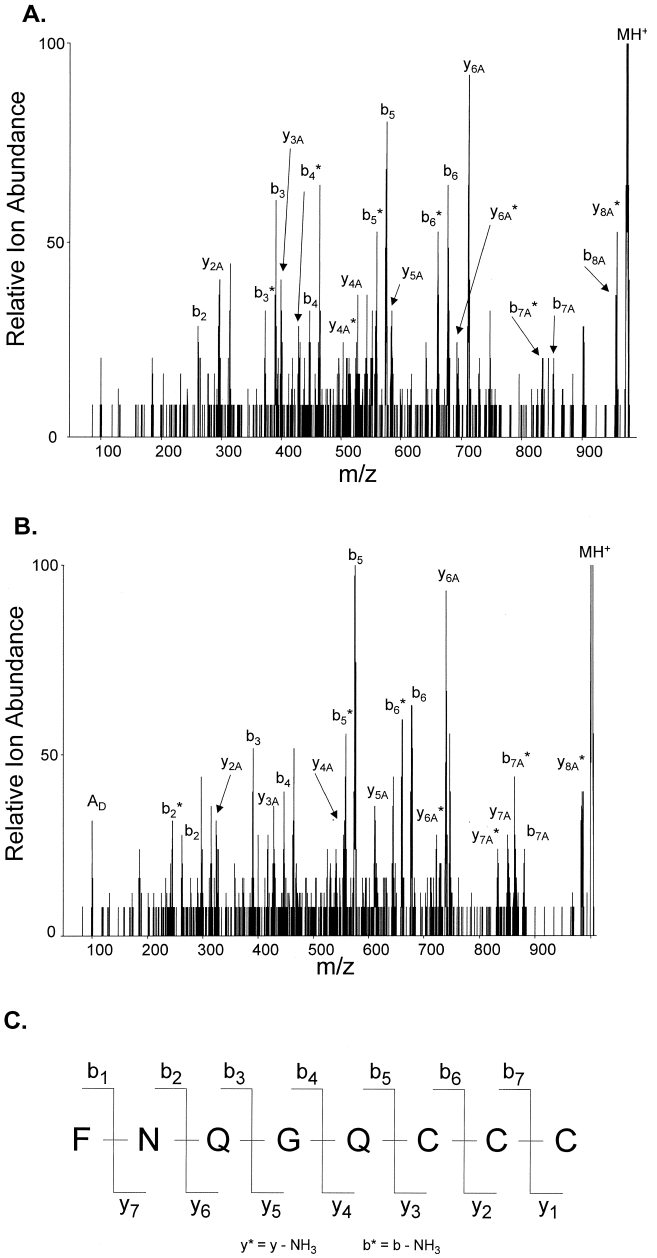

Fig. 4. On-line pepsin digestion-HPLC–MS/MS product ion spectra of adducted peptides (MH^+ = 973.7 and MH^+ = 1001.8). DSF-treated mALDH were infused through the pepsin digestion cartridge, and the resultant peptides were separated by HPLC followed by on-line MS/MS analysis of the *in vivo* drug-modified peptides. (A) Product ion spectrum of peptide of MH^+ = 973.7 from pepsin digestion of *in vivo* disulfiram-inhibited mALDH. The adduct can be characterized as the *N*-ethylcarbamoylation product located on Cys_{302} of peptide $FNQGQC_{301}C_{302}C_{303}$. (B) Product ion spectrum of peptide of MH^+ = 1001.8 from pepsin digestion of *in vivo* disulfiram-treated mALDH. The adduct can be identified as the *N*-diethylcarbamoylation product on Cys_{302} of the peptide $FNQGQC_{301}C_{302}C_{303}$. (C) Representative schematic of how a peptide fragments when subjected to MS/MS analysis. The 'b' series of ions results from fragmentation of the peptide backbone with charge retention at the N-terminus, whereas the 'y' series of ions result from charge retention at the C-terminus. The 'b*' and 'y*' ions are derived from loss of $-NH_3$ from the original 'b' and 'y' ion series. The nomenclature is after that of Roepstorff and Fohlman [32].

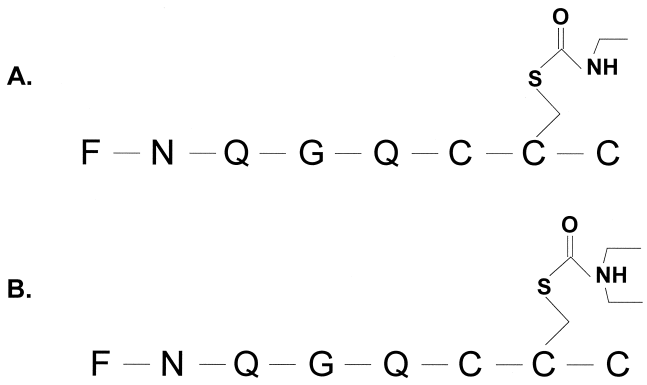

Fig. 5. Structures of *in vivo* adducted peptides. (A) The structure of adducted peptide at $MH^+ = 973.6$ was determined by HPLC–MS/MS as the peptide $FNQGQC_{301}C_{302}C_{303}$ with the *N*-ethylcarbamoyl adduct formed with the thiol on Cys_{302}. (B) The structure of adducted peptide at $MH^+ = 1001.8$ was shown by HPLC–MS/MS as the peptide $FNQGQC_{301}C_{302}C_{303}$ with an *N*-diethylcarbamoyl adduct on Cys_{302}.

ethylcarbamoyl adduct could be detected on glutathione in rat bile following administration of DDC, a known metabolite of DSF [12]. Furthermore, Casida and colleagues have recently reported that *S*-methyl *N*-butylthiocarbamate readily carbamoylates mALDH when administered i.p. to mice [37].

The product ion spectrum of the second adducted peptide, $MH^+ = 1001.8$ (Fig. 4B), also affords an adducted 'y' ion series y_{2A}, y_{3A}, y_{4A}, y_{5A}, y_{6A}, y_{7A}, y_{6A}*, y_{7A}*, and y_{8A}* at *m/z* = 324, 427, 555, 612, 740, 854, 723, 837, and 984, respectively. Adducted 'b' ions b_{7A} and b_{7A}* observed at *m/z* = 880 and 863, respectively, confirmed that adduction had occurred at Cys_{302}. Furthermore, an ion at *m/z* = 100 (marked A_D) indicated the presence of an *N*-diethylcarbamoyl adduct (Fig. 4B). Comparison of this product ion spectrum with that derived from the *in vitro* MeDTC-SO incubation with human recombinant ALDH proved to be almost identical, confirming that the modification was the *N*-diethylcarbamoyl adduction at Cys_{302} of the enzyme active site (Fig. 5B).

4. Discussion

We have shown in this report that DSF is a potent active site inhibitor *in vivo* in rats. Numerous contrary hypotheses have been made in the past with regard to the mode of interaction between ALDH and DSF *in vivo*. It is believed by some that DSF inhibits ALDH by forming a mixed disulfide at the active site [4,6,38], while others report that the inhibition may be due to formation of an intramolecular disulfide bridge [33,39]. In addition, it has been reported that DSF inhibits horse liver mALDH by forming a mixed disulfide, but not at the active site [34]. We have shown definitively that DSF exerts its inhibitory effect *in vivo* in rats by forming covalent adducts on Cys_{302}, one of the three adjacent essential cysteines in the active site of ALDH, and

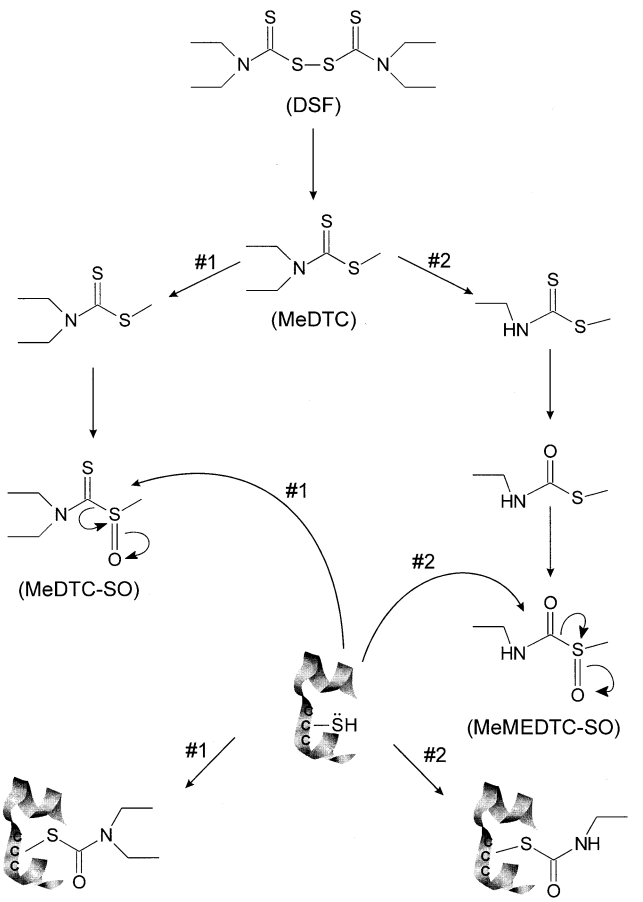

Fig. 6. Proposed metabolic pathway for DSF leading to the formation of the two electrophilic intermediates, MeDTC-SO (pathway 1) and MeMETC-SO (pathway 2). Each metabolite can react with the nucleophilic sulfhydryl group of Cys_{302} to afford inhibited, adducted mALDH protein.

not by forming an intramolecular disulfide bridge. This confirms that Cys_{302} is indeed the catalytic residue of ALDH, as indirectly suggested by previous reports [17,35, 36,40,41]. Irreversible covalent modifications of this residue provide an explanation to the phenomenon where recovery of ALDH activity is dependent on liver protein synthesis [4].

Our experiments determined the identity of the adducts as two carbamoyl derivatives. Hence, based on the chemical structure of the adducts, as well as the absence of a peptide containing a disulfide bridge, we conclude that it is unlikely that DSF is the active inhibitor *in vivo* in rats. Formation of the *N*-diethylcarbamoyl adduct strongly indicates that it was derived from interactions between mALDH and the metabolites of DSF, possibly either MeDTC-SO or MeDTC-SO_2. However, Baillie and coworkers have shown previously that MeDTC-SO_2 reacts readily with glutathione *in vitro* at pH 7.8 [12], while MeDTC-SO is relatively stable at that pH. Therefore, MeDTC-SO_2 may not be sufficiently long-lived in a physiological environment, where glutathione concentration is relatively high, to exert its inhibitory effect on ALDH. Therefore, we propose that it is more likely that MeDTC-SO is one of the active inhibitory agents *in vivo*,

and this possible pathway is summarized in Fig. 6 (pathway 1). The identification of the *N*-ethylcarbamoyl adduct at Cys_{302} was somewhat unexpected and intriguing, since, to our knowledge, no individual phase I *N*-dealkyl metabolites of DSF have been reported. These findings suggest that *N*-dealkylation may be an important pathway in DSF oxidative metabolism, as supported by a previous report [12], and that *N*-dealkylation of *S*-methyldiethylthiocarbamate (MeDTC), a metabolite of DSF, could possibly yield an intermediate that could undergo *S*-oxidation to form *N*-monoethylthiocarbamoyl sulfoxide (MeMETC-SO). This putative metabolite could then readily also react with Cys_{302} of mALDH, as shown in Fig. 6 (pathway 2). In addition, it has been proposed that the *S*-alkyl *N*-monoalkylthiocarbamates may be more potent inhibitors of mALDH *in vivo* than the *S*-alkyl *N,N*-dialkylthiocarbamates [37]. Based on our work and Casida's recent findings [37], we suggest that the potential of the *N*-monoalkyl analogs of DSF, being more efficacious therapeutic entities, is worthy of further investigation.

Acknowledgments

This research was supported by NIH Grant AA09543 (J.J.L.) and Finnigan MAT No. 1 (S.N.). The authors thank Mrs. Diana Ayerhart for her assistance in preparing this manuscript and Dr. W. Stephen Brimijoin for his assistance in administering DSF to the animals.

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