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Inhibition of Human Mitochondrial Aldehyde Dehydrogenase by the Disulfiram Metabolite *S*-Methyl-*N*,*N*-diethylthiocarbamoyl Sulfoxide

STRUCTURAL CHARACTERIZATION OF THE ENZYME ADDUCT BY HPLC-TANDEM MASS SPECTROMETRY

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ABSTRACT. *S*-Methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide (MeDTC-SO) is a known metabolite of the aversion therapy drug disulfiram (DSF). MeDTC-SO is also a potent inhibitor of human mitochondrial aldehyde dehydrogenase (hmALDH) with an IC_{50} of 1.5 μM. Inhibition of the enzyme by MeDTC-SO resulted in the addition of ~100 Da to the molecular mass of the intact protein, as determined by on-line HPLC-electrospray ionization MS (LC-MS). Dialysis of the inhibited protein did not reverse the inhibition, and the molecular mass of 54,533 Da (± 0.01%) remained unchanged, indicating that a covalent modification of the protein had occurred. Proteolytic digestion of hmALDH under basic conditions using trypsin at pH 7.8 revealed that the adduct was base labile. However, treating the adducted protein with endopeptidase-Glu-C at pH 3.7 produced a peptide adduct at $MH^+ = 4924$, tentatively attributable to a carbamoylated peptide. This peptide contains three adjacent cysteines, one of which has been implicated as a key amino acid in the highly conserved active site region of ALDH. A pepsin digestion of hmALDH carried out at pH 3.7 and subsequent LC-MS analysis revealed an ion at $MH_2^{2+} = 501.5$, corresponding to the carbamoylated peptide $FNQGQC_1C_2C_3$. This peptide contains the same adjacent active site cysteines. This latter peptide was subjected to LC-MS/MS, which enabled us to determine that the site of carbamoylation was at Cys_2. The MS/MS product ion data also confirmed the presence of a carbamoyl group as the adduct species. BIOCHEM PHARMACOL **54**;11:1253–1260, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. aldehyde dehydrogenase; disulfiram; alcoholism; enzyme inhibition; tandem mass spectrometry; protein–drug interaction; structural characterization

DSF¶ [bis(diethylthiocarbamoyl) disulfide, trade name Antabuse™] has been employed clinically in aversion therapy of recovering alcoholics for over 40 years [1]. It acts by irreversibly inhibiting hepatic ALDH, resulting in an accumulation of acetaldehyde after ingestion of ethanol [2]. The accumulation of acetaldehyde is associated with vasodilation, tachypnea, and tachycardia with subsequent nausea, vomiting, and hypotension [1]. The parent drug, DSF, is reduced rapidly in the bloodstream by glutathione reductase [3] to produce DDC. This latter compound is subsequently methylated to MeDDC and ultimately oxidized to MeDTC-SO [4, 5]. The sulfoxide has been proposed as the ultimate *in vivo* inhibitor of ALDH [5].

It has been demonstrated recently that the *in vitro* treatment of yALDH with MeDTC-SO results in simultaneous inhibition of the enzyme, as well as an increase in the molecular weight of the protein [6]. These observations are consistent with inhibition of ALDH by the formation of a covalent carbamoyl–protein adduct. However, no direct evidence has been obtained to date on determining the structure and site of adduct formation. In the present work, we utilized a combination of LC-MS and LC-MS/MS to investigate the *in vitro* inhibition of hmALDH by MeDTC-SO. We identified both the active site of inhibition, as well as the structure of the MeDTC-SO-derived adduct.

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¶ *Abbreviations:* DSF, disulfiram; DDC, diethyldithiocarbamate; Me-DTC-SO, *S*-methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide; ALDH, aldehyde dehydrogenase; hmALDH, recombinant human liver mitochondrial aldehyde dehydrogenase; rALDH, rat liver mitochondrial ALDH; yALDH, yeast ALDH; CID, collision-induced dissociation; ESI-MS, electrospray ionization-mass spectrometry; LC-MS, on-line HPLC-electrospray ionization-mass spectrometry; LC-MS/MS, on-line HPLC-electrospray ionization-tandem mass spectrometry; MeDDC, *S*-methyl-*N*,*N*-diethyldithiocarbamate; and DTT, dithiothreitol.

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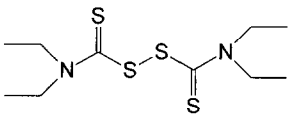
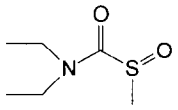


DSF



Me-DTC-SO

MATERIALS AND METHODS

Materials

MeDTC-SO was prepared by the method of Mays *et al.* [7]. DSF (recrystallized twice in ethanol before use, m.p. 71–72°), acetaldehyde, β-mercaptoethanol, and epoxy-activated Sepharose 6B were obtained from the Sigma Chemical Co. (St. Louis, MO); TCPK-treated trypsin, *Staphylococcus aureus V8* (endopeptidase Glu-C) endopeptidase, and pepsin were obtained from Boehringer Mannheim (Mannheim, Germany). The recombinant human mitochondrial and cytosolic cDNA in pT7-7 were gifts from Dr. Henry Weiner (Purdue University, West Lafayette, IN).

Expression of hmALDH in Escherichia coli *and Purification*

The enzyme was isolated using a procedure modified from the method of Jeng and Weiner [8]. Briefly, it consists of growing the cells carrying the plasmid that contains the specific cDNA in 2 L of Terrific Broth supplemented with 100 μg/mL of ampicillin. After harvesting and lysing of cells, the lysate was purified using a DEAE column. The final step of purification utilized a *p*-hydroxyacetophenone affinity column. The final protein concentrations were from 0.5 to 3.5 mg/mL.

hmALDH Activity Assay

The microtiter-based assay was performed as described before [9] with the following modifications: purified recombinant human ALDH was used instead of solubilized rat mitochondria, and rotenone and pyrazole were omitted. The final acetaldehyde concentration was 160 μM for the mitochondrial hmALDH assay. Acetaldehyde and NAD were added together in a 25-μL volume. A concentration–response curve was generated with inhibitor concentrations from 0.1 to 4 μM with a 15-min incubation at room temperature between the addition of inhibitor and the addition of substrate to the enzyme. The reversibility assay was performed by dialyzing inhibited enzyme against 2000 vol. of 50 mM sodium pyrophosphate buffer, pH 8.8, for a total of 30 min as described in detail elsewhere.*

* Lam JP, Mays DC and Lipsky JJ, Manuscript submitted for publication.

Incubation Conditions for Inhibition of hmALDH

One micromolar hmALDH in 20 mM sodium phosphate, 0.1 mM DTT, 1 mM EDTA, and 50 mM NaCl at pH 7.4 was incubated with 40 μM MeDTC-SO at room temperature for 25 min. Forty micromolar DDC, which served as a negative control, was also incubated with hmALDH under identical conditions. Samples were subsequently analyzed directly by LC-MS. hmALDH incubated with MeDTC-SO at pH 8.8 yielded samples that afforded similar results.

Mass Spectrometry

hmALDH was analyzed by LC-MS using a Finnigan MAT 900 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), equipped with a Finnigan MAT designed electrospray interface. LC separations were performed on a Michrom UMA (Michrom Bioresources, Auburn, CA) using a PLRP-S reversed phase, 300 Å pore size, 1.0 × 50.0 mm column (Michrom Bioresources) for the LC-MS analysis of intact proteins. A gradient using mobile phase A (98:2:0.1, by vol., water:acetonitrile:trifluoroacetic acid) and mobile phase B (80:10:10:0.1, by vol., acetonitrile:*n*-propanol:water:trifluoroacetic acid) (from 20–70% mobile phase B) was run for 10 min at a flow rate of 75 μL/min. Twenty microliters of hmALDH solution (~1 μM in 20 mM sodium phosphate, pH 7.4, 0.1 mM DTT, 1 mM EDTA, and 50 mM NaCl) was injected directly onto a 1 × 10 mm reversed phase protein trap inserted as the sample loop of the LC injector. Salts and other hydrophilic matrix components were removed by washing the trap with 300 μL of mobile phase A, prior to diversion of the mobile phase through the trap. A second aliquot of ALDH was analyzed after reaction with 40 μM MeDTC-SO. The entire LC effluent was directed into the electrospray interface and ionized using an electrospray voltage of 3.6 kV, with a 1.6 L/min flow of N_2 as the sheath gas. Ions were detected by a PATRIC™ (Positions and Time Resolved Ion Counter) scanning array detector using an 8% mass window. The estimated error margin associated with the mass measurements in this study was 0.01% of the molecular weight of the protein based upon daily measurement of reference protein standards. Peptides from hmALDH were analyzed by LC-MS and LC-MS/MS using a Finnigan MAT 95Q hybrid mass spectrometer, where MS_1 is a magnetic sector analyzer for selection of precursor ions and MS_2 consists of an octapole collision cell for fragmenting the precursor ion and a quadrupole mass filter for analyzing fragments produced in the collision cell. Sample introduction via LC-MS separation of protein fragments was performed on a Reliasil C-18 reversed-phase HPLC column. A gradient of mobile phase B from 20–70% in 30 min at 50 μL/min was employed. LC-MS/MS experiments were performed using argon as the collision gas at a pressure of 1×10^{-5} mbar, as measured in the quadrupole vacuum chamber. A collision energy of −24 V (lab frame) was used for the fragmentation experiments.

Digestion Conditions

Twenty microliters of either native or MeDTC-SO-adducted hmALDH was diluted with 40 μL of an aqueous solution containing 20 mM ammonium acetate and 1% acetic acid (pH 3.7) and incubated at 37° for 2 hr with either 10% (w/w) *S. aureus* V8 (Glu C) endopeptidase or pepsin. Native or adducted protein was also subjected to trypsin digestion in Tris at pH 8.5. The resultant peptide mixtures were concentrated under vacuum to 20 μL and analyzed by LC-MS under the conditions detailed above.

RESULTS AND DISCUSSION

DSF has been used as an aversion therapy drug for approximately four decades. However, the mechanism of inhibition has still not been directly determined. Hart and Faiman [5] have proposed, but not demonstrated, that MeDTC-SO may be the ultimate inhibitor of ALDH. This is based primarily on the observation that MeDTC-SO has been detected in the plasma of rats given DSF, and it is a more potent inhibitor of ALDH than other known metabolites [5]. Recently, we demonstrated *in vitro* that MeDTC-SO inhibits yeast cytosolic and mitochondrial ALDH, and concomitantly results in a net mass increase of ~100 Da for the protein, as determined by ESI-MS [6]. Hence, we were interested in determining the precise mechanism and site of action of MeDTC-SO on ALDH.

Inhibition of hmALDH by MeDTC-SO

We have shown previously that the IC_{50} of MeDTC-SO on rat liver mitochondrial low K_m ALDH (rALDH) is 0.93 ± 0.04 μM, as compared with 7.4 ± 1.0 μM for the parent drug DSF [7]. Furthermore, addition of glutathione (50–1000 μM) to the detergent-solubilized mitochondria did not alter the inhibition potency of MeDTC-SO on rALDH. Subsequently, we measured the inhibition effect of MeDTC-SO on the activity of hmALDH at pH 8.8.* (This pH value is optimal for ALDH activity in the more basic medium of the mitochondrial matrix [10]). It was shown that Me-DTC-SO is a potent inhibitor of hmALDH with an IC_{50} value of 1.5 μM. It was also demonstrated that by dialyzing the inhibited enzyme, the *in vitro* inhibition of hmALDH still remains, indicating that the interaction is irreversible. This strongly indicates that the adduct formed is covalently bound to the enzyme.

Simultaneous Enzyme Inhibition Assay/LC-MS Analysis of hmALDH Reacted with MeDTC-SO

The traditional approach to studying protein–drug interactions includes a number of indirect techniques such as radiolabel incorporation, site-directed mutagenesis, and immunofluorescence. However, we have demonstrated that the combined approach of enzyme inhibition assay coupled with ESI-MS analysis of the protein–drug adduct affords a very powerful approach to unequivocally and directly determine covalent modification of the protein [6]. In particular, we demonstrated that addition of MeDTC-SO to yALDH results in a causal link between inhibition of the enzyme and a concomitant increase of ~100 Da, as determined by ESI-MS. We postulated that this could be rationalized due to carbamoylation at the active site of yALDH.

A similar approach was adopted for the analysis of MeDTC-SO inhibited with hmALDH. However, we used LC-MS in order to improve specificity and accuracy of molecular weight determination. Initially, native hmALDH was subjected to LC-MS and a molecular mass (M_r) of 54,432 Da (±0.01%) was determined, as shown in Fig. 1A. The protein also possessed enzyme activity (100%). Subsequently, independent aliquots of this solution containing 1 μM hmALDH were reacted with 40 μM MeDTC-SO and 40 μM DDC, both at room temperature for 25 min at pH 7.4. The latter reaction served as a negative control experiment. Both the MeDTC-SO- and DDC-treated samples were subjected to simultaneous enzyme activity and LC-MS analysis. The latter sample still retained 100% enzyme activity and an M_r of 54,432 Da (data not shown). However, the MeDTC-SO-treated enzyme exhibited <10% activity compared with the control, as well as a concomitant increase in M_r of ~100 Da to 54,533 Da (±0.01%) as determined by LC-MS (Fig. 1B). Approximately 80% of the native protein was adducted, based on peak area measurements of M_r 54,432 (Fig. 1A) and 54,533 (Fig. 1B). This increase of ~100 Da in the molecular mass of hmALDH is consistent with a single carbamoylation of the peptide/protein by MeDTC-SO as noted previously for yALDH [6] and for glutathione [11].

Stability of hmALDH-MeDTC-SO Adduct

We have shown previously that the intact protein–drug adduct produced from incubation of MeDTC-SO with yALDH is stable at room temperature within the pH range of 2.5 to 9.5 [6]. However, treatment of adducted yALDH with 1 mM DTT at pH 9 resulted in recovery of enzyme activity and a loss of ~100 Da. We proposed that this occurred because of the increased nucleophilic character of the thiolate anion of DTT, present at high pH (pK_a DTT-SH ~8.5). It was postulated that the basic pH facilitates nucleophilic attack by the thiolate anion on the carbonyl group of the thioester linkage of the carbamoyl adduct, resulting in acyl cleavage to afford the native yALDH.

The intact protein–adduct formed from the reaction of hmALDH with MeDTC-SO was also found to be stable at room temperature at physiological pH (~7.2). However, it was important to determine the stability of the adduct over an increased pH range when the hmALDH either had been denatured or was subjected to proteolytic digestion. This

* Lam JP, Mays DC and Lipsky JJ, Manuscript submitted for publication.

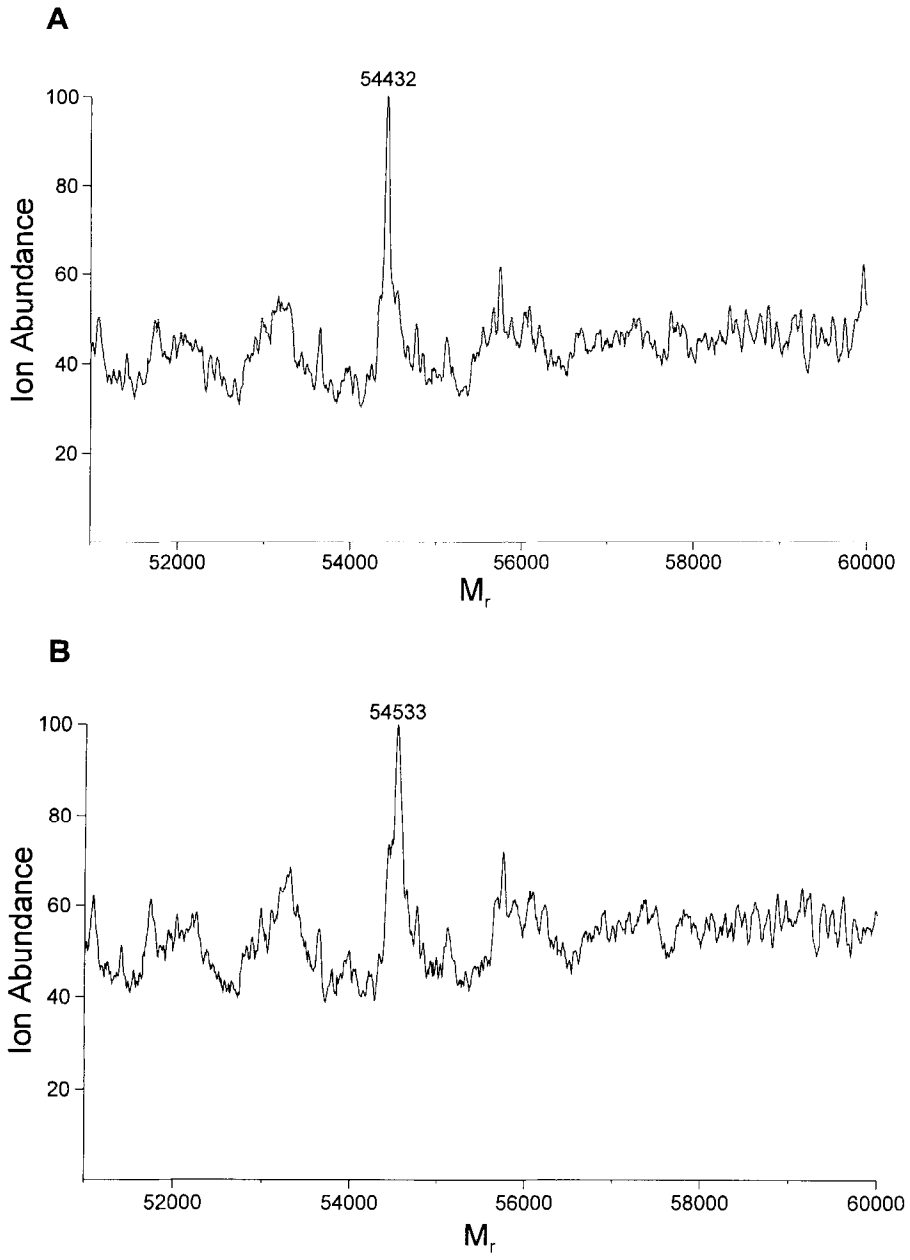


FIG. 1. LC-MS analysis of hmALDH performed in conjunction with enzyme inhibition assay. (A) Positive ion LC-ESI-MS of native hmALDH. (B) Positive ion LC-ESI-MS of hmALDH incubated *in vitro* with MeDTC-SO. A net mass shift of ~+100 Da was observed, as compared with the native enzyme.

was necessary since in order to determine both the site of adduction and structure of the adduct by LC-MS/MS, peptide fragments in the mass range ~500–3000 Da had to be generated [12].

In the intact protein, the adduct can be stabilized by the overall conformation and localized buffering capability of amino acid side chains of the protein. This phenomenon has been reported previously for a hemoglobin-benzo-[a]pyrene diolepoxide ester adduct [13, 14] which, upon trypsin digestion, resulted in the peptide adduct ester being readily hydrolyzed at both basic and acidic pH values [13]. Therefore, the possibility that MeDTC-SO had formed an adduct via a thioester-like linkage with hmALDH led us to favor acidic pH enzyme digestion conditions. Thioesters are more prone to facile base hydrolysis but are much less susceptible to acid hydrolysis [15].

Initially, we subjected both native and MeDTC-SO-treated hmALDH to endopeptidase Glu-C digestion at 37° for 2 hr in 20 mM NH_4OAc and 1% acetic acid (pH 3.7). Each digest was subjected independently to on-line LC-MS, and these analyses revealed a complex mixture of peptides. In the native protein digest, a peptide was detected with multiply charged ions at MH_3^{3+} = 1609.3 and MH_4^{4+} = 1207.0, corresponding to an M_r of 4825.0 Da (Fig. 2A). This molecular mass is consistent with an endopeptidase Glu-C generated peptide spanning the ac-

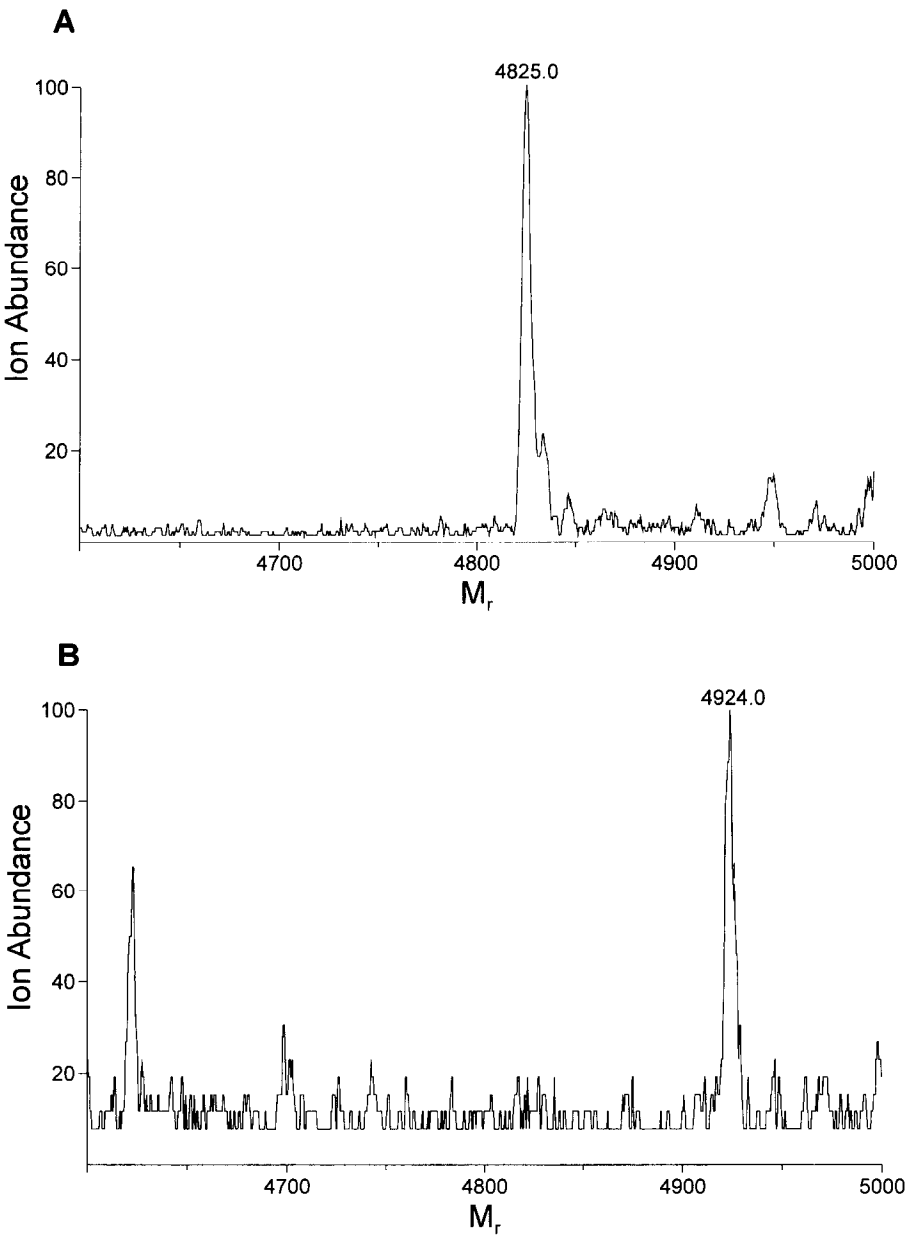


FIG. 2. LC-MS analysis of hmALDH after proteolytic digestion with endopeptidase Glu-C, carried out at acidic pH 3.7. (A) Deconvoluted ESI-MS spectrum of ions between *m/z* 1190 and 1900 after endopeptidase Glu-C enzyme digestion of native hmALDH. A molecular ion (M_r) at 4825.0 was observed corresponding to LGGKSPNIIMSDADMDWAVEQAHFALFFNQGQCCCAGSRTFVQE. (B) Deconvoluted ESI-MS spectrum of ions between *m/z* 1190 and 1900 after endopeptidase Glu-C digestion of hmALDH incubated *in vitro* with MeDTC-SO. A mass shift of +99 Da was observed, affording a molecular ion (M_r) at 4924.0.

tive site region of hmALDH and comprising a sequence of LGGKSPNIIMSDADMDWAVEQAHFALFFNQGQCCCAGSRTFVQE [16].

LC-MS analysis of the endopeptidase Glu-C digest of hmALDH reacted with MeDTC-SO also revealed multiply charged ions at *m/z* 1609.3 and 1207.0 corresponding to the native, unmodified peptide (data not shown). However, the relative ion abundance of these responses was <10% of that observed for the native protein digest. Furthermore, abundant ions were also observed at *m/z* 1642.3 and 1232.0, which were not present in the native protein digestion. These ions correspond to the MH_3^{3+} and MH_4^{4+} multiply charged ions of the endopeptidase Glu-C derived peptide described above but modified by the overall addition of a mass of +99 Da, affording an M_r of 4924.0 (Fig. 2B). This can be rationalized by carbamoylation of the native peptide brought about by the reaction of MeDTC-SO with the protein. Furthermore, this peptide contains the three adjacent cysteines that have been implicated in the highly conserved active site region of ALDH [17, 18]. No other modified peptide was detected in the Me-DTC-SO-treated hmALDH enzyme digest.

The same LC-MS analyses were also carried out on both native and adducted hmALDH after digestion with trypsin. In this case, basic pH digestion conditions were used where each protein was dissolved in 100 mM Tris at pH 8.5 and incubated at 37° for 2 hr. The LC-MS analyses of both native and adduct hmALDH digests were almost identical (data not shown). There was no evidence for a carbamoylated peptide in adducted hmALDH digest. This finding is consistent with the fact that the carbamoyl adduct is base

labile upon disruption of the native conformation of the protein after enzyme digestion.

LC-MS and LC-MS/MS Analysis of Pepsin Enzyme Digest of MeDTC-SO-Treated hmALDH

LC-MS MAPPING. The pH-dependent lability of the peptide–MeDTC-SO adduct coupled with the molecular mass range constraint of the peptide needed for efficient MS/MS sequence analysis necessitated that an enzyme digest should: (a) be carried out using an enzyme with a V_{max} that is optimum at acidic pH, and (b) produce peptides in the molecular mass range of ~500–3000 Da.

Unfortunately, the use of endopeptidase Glu-C produced an adducted peptide (M_r = 4924.0) that was too large to efficiently obtain amino acid sequence and adduct structural information. However, pepsin is a proteolytic enzyme that cleaves at the C-terminal side of hydrophobic amino acids such as phenylalanine, methionine, leucine, isoleucine, and tryptophan [19]. The enzyme is particularly efficient at hydrolyzing amide bonds located in hydrophobic amino acid cluster regions. Typically, a pepsin digestion of a protein produces peptides in the 500–2500 Da mass range, which is ideal for LC-MS/MS analysis. Furthermore, it is an enzyme that has maximum proteolytic activity in acidic solutions. Hence, a pepsin digestion of MeDTC-SO-inhibited hmALDH was carried out under conditions identical to those used for endopeptidase Glu-C at pH 3.7 and was incubated for 2 hr at 37°. The resulting peptide digest was subjected immediately subjected to LC-MS mapping analysis, and a plethora of peptide responses were observed (data not shown). On careful inspection, an ion at m/z 501.5 (retention time 18.9 min) was detected which could tentatively be ascribed to the doubly charged ion (MH_2^{2+}) of the carbamoylated peptide FNQGQCCC located at the active site of the enzyme. An ion at m/z 1002 at the same retention time was also detected, which ostensibly could be the singly charged (MH^+) ion of the same adducted peptide. However, a subsequent LC-MS analysis of the same enzyme digest at higher resolution in MS_1 (~1500) revealed that this ion was doubly charged, corresponding to an MH^+ = 2001, which could tentatively be assigned to the peptide sequence NRLADLIERDRTY LAAL present in hmALDH [16]. There was no detectable presence of the unadducted peptide FNQGQCCC, which has a protonated molecular ion (MH^+) at 902.3.

A pepsin digest of native hmALDH, carried out under identical conditions as described for adducted protein, revealed the same complex pattern of peptides. There was no detectable presence of an ion at MH_2^{2+} = 501.5, corresponding to the carbamoylated peptide FNQGQCCC. However, a prominent ion at m/z = 902.3 corresponding to the MH^+ of native FNQGQCCC at a retention time of 15:06 min was observed. The ion at m/z 1002 corresponding to the MH^{2+} of the peptide NRLADLIERDRTYLAAL was also clearly detectable in the pepsin digest of the native hmALDH. Both the MeDTC-SO-treated and native hmALDH were subjected to pepsin digestion followed by LC-MS analysis on four separate occasions. In all cases, the results were identical to those described above. The LC-MS mapping experiments indicated that the peptide FNQGQ CCC present at the active site region of hmALDH had been modified by an overall increase in molecular mass of +99 Da. This modification had resulted in the inhibition of hmALDH activity, and all the pepsin data were consistent with that previously obtained for an endopeptidase Glu-C digestion of hmALDH (see above). Furthermore, no other modified peptide was detected in the LC-MS analysis of this peptide mixture.

LC-MS/MS SEQUENCE ANALYSIS. In many instances, the primary sequence 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 tandem mass spectrometry (MS/MS) has played a key role in determining the nature and site of adduction after the protein has been subjected to proteolytic digestion [20–22]. We have previously discussed optimal conditions for the CID of peptides [23]. Employing similar collision conditions, the doubly charged precursor ion at MH_2^{2+} = 501.5, from the pepsin digest of MeDTC-SO-adducted hmALDH, was subjected to LC-MS/MS. The resulting product ion spectrum is shown in Fig. 3. A series of singly charged (MH^+) 'b' (b_2–b_5, at m/z 262, 390, 447, and 575, respectively); b* (b_2^*–b_6^* at m/z 245, 373, 430, 558, and 661, respectively); and 'y' (y_3, y_5, y_6 at m/z 427, 612, and 740, respectively) indicated that the partial unmodified sequence of FNQGQC was present in the adducted peptide. This was confirmed by the presence of a series of doubly charged ions (MH_2^{2+}) for b* (b_3^*–b_6^* at m/z 187, 216, 280, and 331, respectively) and 'y' (y_3–y_8 at m/z 214, 278, 307, 371, 428, and 501, respectively) CID fragmentations. These data strongly suggested that adduction of the peptide $FNQGQC_1C_2C_3$ had occurred at either cysteine Cys-2 or Cys-3. However, specific evidence that adduction had occurred at Cys-2 was indicated by singly charged (MH^+) product ions at m/z 307 (y_2^*), 324 (y_2), and 852 (a_7), as well as doubly charged (MH_2^{2+}) product ions at m/z 427 (a_7), 478 (a_8), 418 (a_7^*), and 432 (b_2^*).

To unequivocally determine the site of adduction of the pepsin-produced adducted peptide, the entire digest of MeDTC-SO-adducted hmALDH was subjected to methanol/HCl reaction conditions. This readily and efficiently methylates both the C-terminus and amino acid side chain carboxylic acids. It has been used to improve the 'y' series ions in positive ion CID MS/MS spectra [23]. Subsequently, the methylated digest was subjected to LC-MS mapping using identical procedures as described above. A doubly charged (MH_2^{2+}) ion at m/z 508.2 was detected in the methylated MeDTC-SO-inhibited enzyme at a retention time of 21.1 min, corresponding to the adducted peptide FNQGQCCC(=O)OMe. This response was not observed in LC-MS analysis of the methylated native hmALDH digest (data not shown). Subsequently, the ion at m/z 508.2 was subjected to CID using LC-MS/MS, and the product

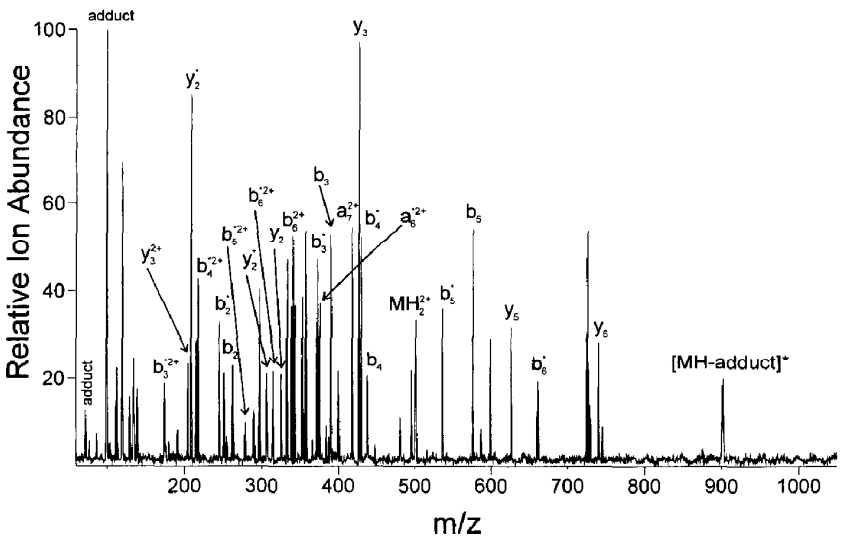
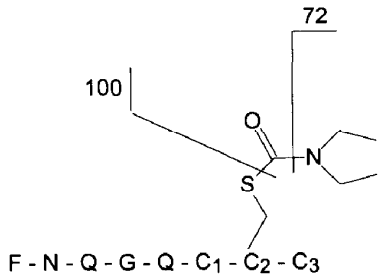


FIG. 3. LC-MS/MS product ion spectrum of adducted peptide (MH_2^{2+} = 501.5) derived from pepsin digestion of MeDTC-SO-treated hmALDH. Nomenclature modified from Ref. 24.

ion spectrum is shown in Fig. 4. It should be noted that a singly charged (MH^+) 'b' series (b_2–b_6) at m/z 262, 390, 447, 575, and 678 again confirm that amino acid residues 1–6 are not modified. As predicted, the 'y' series product ions were enhanced when the C-terminus was methylated and a number of singly charged (MH^+) y_1–y_3 ions at m/z 136, 338, and 441, respectively, were observed. This unequivocally locates the adduct at Cys-2 in the peptide FNQGQC$_1$C$_2$C$_3$.

Once the amino acid sequence of the peptide, as well as the site of adduction, was determined, the product ion spectrum (Fig. 3) was analyzed subsequently for information concerning the structure of the adduct. Inspection of the product ion spectrum reveals an ion at m/z 902.3 (MH^+) corresponding to the native peptide FNQGQCCC after loss of the intact adduct. This is consistent with the presence of a carbamoyl-peptide adduct since Baillie and coworkers [11] have reported previously the facile loss of the carbamoyl group from a glutathione-carbamoyl adduct after CID. Furthermore, product ions at m/z 72 and 100 observed in the product ion spectrum (Fig. 3) have been reported previously to be highly characteristic of a carbamoyl group [11, 25]. The specific fragmentation pathways of the carbamoyl group that give rise to these ions are shown below, in the structure of the carbamoylated-peptide adduct derived from the pepsin digestion of MeDTC-SO-treated hmALDH.



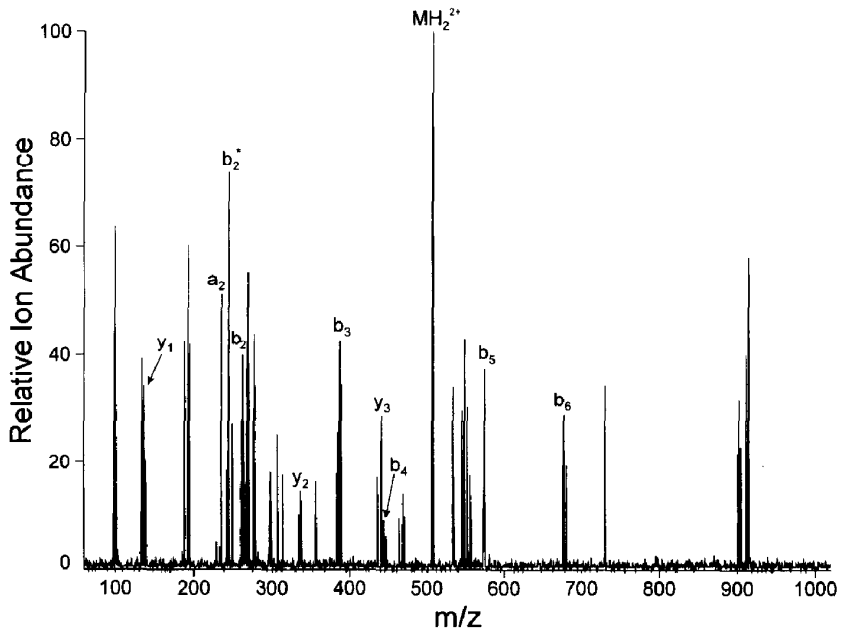


FIG. 4. LC-MS/MS product ion spectrum of methylated adducted peptide (MH_2^{2+} = 508.2). The entire pepsin digest of MeDTC-SO-treated hmALDH was subjected to treatment with MeOH/HCl to effect C-terminal and acidic amino acid side-chain methylation.

In conclusion, we have developed a strategy that readily determines the chemical structure of a pH-labile enzyme–drug covalent adduct. We use LC-MS to directly analyze the intact native protein, as well as the adducted protein. This analysis, in conjunction with simultaneous enzyme inhibition assay, affords a powerful approach to show causal relationship between adduct formation and cessation of protein activity. Furthermore, using LC-MS mapping of the protein after proteolytic digestion gives rapid information on both the stability of the peptide adduct, as well as which peptide has been modified. LC-MS/MS is then used to identify unambiguously the site of adduct formation, as well as the structure of the adduct. Based on this strategy, we have, to our knowledge, for the first time directly demonstrated that Cys-2 of the peptide present at the active site of hmALDH is carbamoylated after reaction with MeDTC-SO. This results from nucleophilic attack of the cysteine sulfur at the carbamoyl of MeDTC-SO. This strongly suggests that the interaction of MeDTC-SO with a specific Cys amino acid side-chain at the active site of hmALDH is a major mode of inhibition of this enzyme by the compound.

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