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# Inhibition of Aldehyde Dehydrogenase by Disulfiram and Its Metabolite Methyl Diethylthiocarbamoyl-Sulfoxide

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**ABSTRACT.** Disulfiram (DSF) is presently the only available drug used in the aversion therapy of recovering alcoholics. It acts by inhibiting aldehyde dehydrogenase (ALDH), leading to high blood levels of acetaldehyde. The *in vitro* inhibition of ALDH by DSF and its metabolites was systematically studied by combined enzyme inhibition assay with direct molecular weight determination of the same sample using electrospray ionization–mass spectrometry (ESI–MS). Enzyme activity was measured after incubating yeast ALDH (yALDH) with excess concentrations of DSF, methyl diethyldithiocarbamate (MeDDC) and methyl diethylthiocarbamoyl-sulfoxide (MeDTC-SO) and then subjected to analysis by ESI–MS. Addition of DSF resulted in complete enzyme inhibition; however, ESI–MS analysis demonstrated no discernible shift in molecular weight, indicating that no intermolecular adduct was formed with the protein. Treatment of yALDH with MeDTC-SO also completely abolished yALDH activity with a concomitant increase of +≈100 Da in the molecular mass of the enzyme. This indicated formation of a covalent carbamoyl protein adduct. Furthermore, the effects of dithiothreitol (DTT) were examined on samples of inhibited protein *in vitro*. At pH 7.5, DTT completely reversed inhibition after DSF treatment. yALDH inhibited by MeDTC-SO could not be recovered by DTT at pH 7.5, but at pH 9 the enzymic activity was fully restored and a mass loss of ~100 Da was noted. This observations are consistent with mechanisms where inhibition of yALDH by DSF *in vitro* involves oxidation of the active site, whereas MeDTC-SO forms a covalent adduct with the protein *in vitro* resulting in cessation of enzyme activity. BIOCHEM PHARMACOL 53;4:511–518, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** mass spectrometry; enzyme inhibition; aldehyde dehydrogenase; disulfiram; alcoholism; protein modification

DSF** (tetraethylthiuram disulfide, trade name Antabuse) is a compound used clinically in the aversion therapy of recovering alcoholics. It acts by irreversibly inhibiting the hepatic enzyme ALDH, resulting in an accumulation of acetaldehyde after ingestion of alcohol [1]. The *in vivo* metabolism of DSF is well characterized and described and is summarized in Fig. 1. The parent drug is reduced rapidly in the bloodstream to DDC, which is subsequently methylated and ultimately oxidized to MeDTC-SO [1, 2]. This latter compound has been reported to be the ultimate inhibitor of ALDH [3].

It is well established that an active metabolite of DSF irreversibly inhibits ALDH via covalent modification of the active site (see, for example, Refs. 1 and 3). However, previous studies have presented an ambiguous model of what is actually occurring. Neims *et al.* [4] proposed that inhibition occurs due to the formation of a mixed disulfide between a cysteine-SH group and DDC. However, Strömme [5] reported that the feeding of [$^{35}$S]DSF to rats resulted in "hardly any mixed disulfide formation" based on negligible incorporation of label on isolated ALDH. Subsequently, Vallari and Pietruszko [6] also demonstrated that no incorporation of radiolabel occurred when [$^{14}$C]DSF was reacted *in vitro* with isolated human cytoplasmic ALDH. They suggested that this could be explained by invoking oxidation of proximal cysteines at the active site to produce a disulfide bridge.

In the present work, we describe a novel method employing simultaneous ALDH enzyme inhibition assay coupled with direct molecular weight determination of na-

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** *Abbreviations:* DSF, disulfiram; ALDH, aldehyde dehydrogenase; yALDH, yeast aldehyde dehydrogenase; DDC, diethyldithiocarbamate; MeDDC, *S*-Methyl-*N*,*N*-diethylthiocarbamate; MeDTC, methyl diethylthiocarbamate; MeDTC-SO, *S*-Methyl-*N*,*N*-diethylthiocarbamoyl sulfoxide; DDT, 1,4-dithiothreitol; and ESI–MS, electrospray ionization–mass spectrometry.

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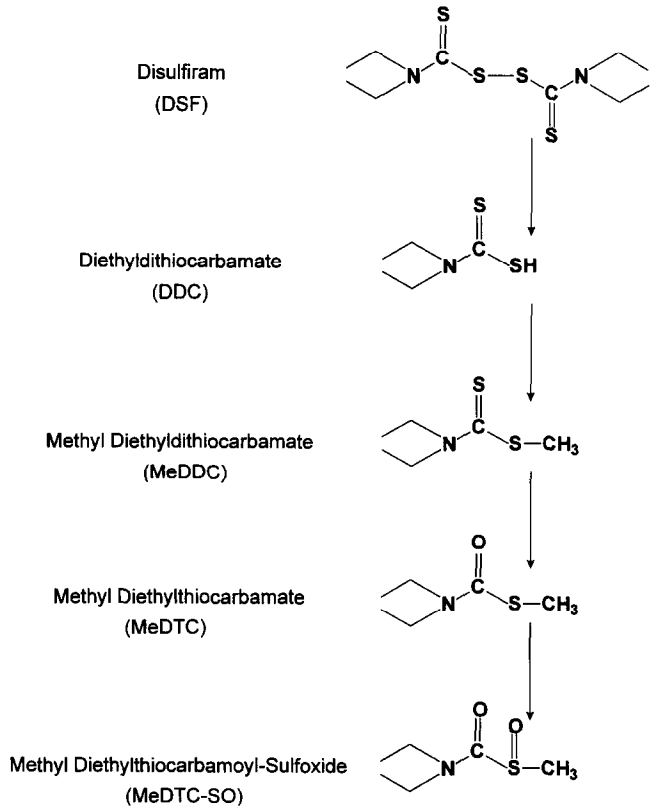


**FIG. 1. Proposed metabolic pathway for the biotransformation of the parent drug DSF to MeDTC-SO.**

tive and adducted proteins using ESI–MS. In particular, by using this approach, it allowed us to directly investigate the *in vitro* inhibition of yALDH by DSF and also MeDTC-SO.

## MATERIALS AND METHODS

### *Materials*

DSF (recrystallized twice before use, m.p. 71–72°), rotenone, and pyrazole were purchased from Sigma (St. Louis, MO). yALDH [aldehyde:NAD(P) oxidoreductase, EC 1.2.1.5; specific activity = 51 U/mg enzyme protein] isolated from *Saccharomyces cerevisiae*, NAD (grade I free acid, 100%), NAD (grade II, 98% pure), Tris–HCl, and Tris base were obtained from Boehringer Mannheim (Mannheim, Germany). 5′-AMP Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). Acetaldehyde, DTT, and sodium pyrophosphate were obtained from Aldrich (Milwaukee, WI). Acetonitrile, methanol (Burdick & Jackson, UV grade), and ethanol (redistilled before use) were purchased from Baxter (McGaw Park, IL).

### *Chemical Syntheses*

MeDTC was synthesized as described by Hart *et al.* [7]. MeDTC-SO was synthesized by reacting MeDTC with 1.2 equivalent of *m*-chloroperbenzoic acid at 0° overnight in chloroform. It was subsequently purified by silica gel chromatography (230–400 mesh) using 10% methanol in chloroform. The yield was 48% and purity was ≥99% by HPLC. Characterization by elemental analysis, $^1$H-NMR, and mass spectral data have been reported previously elsewhere [8].

### *FPLC Purification of Yeast ALDH*

ALDH from Baker's yeast was supplied by Boehringer Mannheim and stated to be "≥99% pure based on activity." This protein was further purified to a single band by SDS–PAGE (Coomassie Blue and Silver stain) using affinity chromatography in a column packed with 5′-AMP Sepharose (column dimensions were 1.6 cm diameter × 3 cm height; void volume ~2.5 mL). For purification of yALDH at pH 7.5, the column equilibration buffer was 0.02 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA. The buffer used to elute the protein from the column was identical to the equilibration buffer with the addition of 10 mM NAD (grade II). For purification of yALDH at pH 9, the column equilibration buffer and elution buffer were prepared as described for the 0.02 M sodium phosphate buffer, pH 7.5; however the buffer was comprised of 0.1 M Tris, pH 9, containing 1 mM EDTA. All buffers and samples were filtered through a 0.2 mm filter and a 45 μm filter, respectively, prior to application to the FPLC system (Pharmacia LKB). The entire FPLC system, including columns, fraction collector, and buffer, was maintained in a cold room at 4°.

A 0.5-mL aliquot of the 20 mg/mL yALDH solution in equilibration buffer was applied directly to an injection loop (0.5 mL total volume) for sample introduction. The FPLC run was as follows: column was equilibrated isocratically with buffer at a flow rate of 0.5 mL/min for 16 mL; elution of protein adsorbed to 5′-AMP matrix proceeded during a gradient of 0–10 mM NAD from 17 to 30 mL; NAD concentration was held at 10 mM from 30 to 35 mL to ensure total elution of protein; then the NAD concentration was reduced to 0 mM (equilibration buffer) from 36 to 40 mL; finally, the column was re-equilibrated from 41–50 mL. Fractions were collected from 23 to 32 mL, and subsequently analyzed for yALDH activity and protein concentration. Activity corresponding to yALDH was found in fractions 26–30 when yALDH was purified at pH 7.5, and in fractions 23–27 for the pH 9 purification procedure. This indicated that yALDH is displaced from the 5′-AMP Sepharose column by approximately 4 mM NAD at pH 7.5, and approximately 2 mM NAD at pH 9. The yield of recovered activity obtained by purification of yALDH using 5′-AMP Sepharose chromatography was ≥88%.

Protein concentrations were determined by the kinetic BCA protein assay (Pierce, Rockford, IL) adapted for a microtiter plate reader using BSA to prepare protein standards. A standard curve of BSA was prepared in buffer containing NAD to emulate the concentration of NAD that displaces ALDH from the 5′-AMP Sepharose column (†4 mM NAD) to ensure that NAD did not interfere with the BCA kinetic protein quantitation.

### *yALDH Assay*

The activity of yALDH was assayed with a microplate reader (Molecular Devices, Menlo Park, CA) at 22° by following the formation of NADH spectrophotometrically at 340 nm [9]. Except where noted, the assays contained the following: 50 mM sodium pyrophosphate buffer (pH 8.8), pyrazole (100 μM final concentration, added in 10 μL of 50 mM sodium pyrophosphate buffer), rotenone (2 μM final concentration, added in 1.4 μL of methanol); and yALDH (added in a 25-μL aliquot of respective sodium phosphate buffer). The preincubation volume was 700 μL. Samples were dispensed as triplicate aliquots of the preincubation mixture into the microplate wells, and NAD (2.4 mM final concentration in 25 μL of sodium pyrophosphate buffer) was added. The reaction was started by addition of the substrate acetaldehyde (2.7 mM final concentration), and the entire plate was mixed prior to data acquisition. The order of addition of inhibitor, substrate, and cofactor was the same, unless noted. Data acquisition was initiated within 1 min of addition of the substrate to the mixture in the microtiter plate wells.

yALDH activity after *in vitro* reaction with DSF, MeDDC, and MeDTC-SO was studied using the same reaction conditions as described above for the yALDH assay with the exception of adding inhibitor. All inhibitors were prepared in methanol. Unless an additional incubation period is specified, yALDH was exposed to inhibitor for approximately 15 min at 22° while preparing the microplate for reading. In inhibition experiments done prior to ESI–MS analysis (adding drug in excess of the $IC_{50}$ value), the final concentration of inhibition was as follows: $[DSF]_{final}$ = 20 μM, $[MeDDC]_{final}$ = 800 μM, and $[MeDTC\text{-}SO]_{final}$ = 300 μM.

### *DTT Recovery of Inhibited yALDH*

We subjected the reaction mixtures containing yALDH and each of the inhibitors to DTT treatment in order to examine the effects of a reducing agent on the inhibited protein and/or the potential resulting adducts. Identical samples of purified enzyme without inhibitor served as controls for each respective pH value. After samples were reacted with DSF, MeDDC, or MeDTC-SO, they were kept on ice during a 1 h time–course during which yALDH activity was determined at 0, 20, 40, and 60 min both with and without the addition of DTT to separate reaction tubes (prepared as a 100 mM stock solution in 0.05 M sodium pyrophosphate buffer, pH 8.8) to a final concentration of 1 mM. Activity of each yALDH sample was determined as described above except that the enzyme purified at pH 7.5 was assayed in sodium pyrophosphate with the pH adjusted to pH 7.5, while yALDH activity of pH 9 samples was assayed in sodium pyrophosphate, pH 9. Subsequent to activity analysis, samples were subjected to analysis by ESI–MS.

### *Sample Preparation Prior to Analysis by Mass Spectrometry*

Prior to introduction of the sample into the mass spectrometer, samples were preconcentrated and desalted by absorbing them on a reversed phase protein trap cartridge with a bed size of 1 × 10 mm (Michrom BioResources, Auburn, CA). The cartridge was mounted on the injection loop of a Rheodyne 7725 injector valve. Samples (50–500 mL) were loaded onto the microcolumn (trap cartridge) via syringe, and were washed with $H_2O$:$CH_3CN$:acetic acid (98:2:2) to remove excess salts, drugs, and other buffer components/contaminants. Samples containing ALDH protein were eluted from the microcolumn by flushing with $CH_3CN$:$H_2O$:acetic acid (60:40:2) where the liquid flow was delivered by a Harvard Apparatus 22 syringe pump (South Natick, MA) at a flow rate of 10 μL/min for off-line collection, or 5 μL/min for on-line injection directly into the mass spectrometer. Samples off-line were collected from 1.5 to 6.5 min after injection.

### *Mass Spectrometry*

Analyses were carried out on either a Finnigan MAT 900 mass spectrometer (Bremen, Germany) which is a magnetic sector instrument of EB configuration (where E is an electrostatic analyzer and B is a magnet) or a Finnigan MAT 95Q hybrid mass spectrometer (Bremen, Germany) of $BEQ_1W_2$ configuration (where B is the magnet, E is the electric sector, $Q_1$ is a radio frequency (rf)-only octapole collision cell, and $Q_2$ is a mass filter quadrupole). Samples were introduced at a flow rate of approximately 0.5 μL/min (10 psi) from a pressurized sample infusion bomb previously described [10]. Samples were ionized by electrospray ionization using a Finnigan MAT (Bremen, Germany) electrospray ion source operated in the positive ion mode using a sheath liquid of $CH_3CN$:$H_2O$:acetic acid (60:40:2) flowing at a rate of 2 μL/min. The capillary was maintained at 200°. The operating resolution of the instruments was varied between *ca.* 1000 and 2500, scanning over the $m/z$ range 700–2000 Da at a rate of 10–20 sec/decade. Ions were detected by a standard electron multiplier. 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.

## RESULTS AND DISCUSSION

yALDH was selected as a model protein for method development and mechanistic studies on the inhibition of the enzyme by DSF for the following reasons: (1) it is commercially available; (2) although it only has ~60–70% homology with other mammalian ALDHs, the active site region is actually 95–100% homologous [11]. ESI–MS analysis afforded the observation that the sample of "pure" yALDH obtained from Boehringer Mannheim was actually a mix-

ture of two component proteins which co-purified by 5'-AMP Sepharose affinity chromatography, and appeared as a single band by SDS–PAGE. Although it was not possible to resolve these protein components by SDS–PAGE (due to the near equivalence in molecular weight), the presence of two independent proteins was demonstrated by isoelectric focusing PAGE (IEF–PAGE, data not shown). The molecular mass of these proteins was determined to be 53,960 and 54,095 Da (±5 Da) by ESI-MS analysis.

### *Characterization of Native yALDH*

The predicted, average molecular mass for the cytoplasmic form of the yALDH is 54,090 Da [12]. This includes the mass of the N-terminal acetate known to occur on some cytoplasmic forms. The ESI–MS analysis of one of the yALDH components indicated that the actual observed molecular mass was 54,095 ± 5 Da, tentatively identifying it as cytoplasmic yALDH.

Since it is reported that the cytoplasmic forms of ALDH tend to be N-terminally acetylated [12] which ultimately prevents analysis by Edman degradation, the two yALDH component proteins were subjected to Edman sequence. As predicted, the result of 13 cycles of Edman afforded sequence information for only one protein in the sample mixture. When used as a search query in the protein database of Genbank, the 13 amino acid sequence obtained by Edman analysis was homologous with only one known protein sequence, identified as mitochondrial yALDH (Genbank accession number U18814). This suggested that the latter component of the protein mixture was an isoform of mitochondrial yALDH. Furthermore, the molecular mass determined by ESI–MS for the Boehringer yALDH (53,960 ± 5 Da) was very similar to the predicted molecular mass (54,954 Da) for the truncated mitochondrial yALDH described in the protein database.

From the observed data, the two component proteins of the yALDH mixture appear to represent the cytoplasmic and mitochondrial isoforms of the enzyme. This is noteworthy since yALDH supplied by Boehringer Mannheim is not subjected to subcellular fractionation as a part of their proprietary purification procedure, which is consistent with our observation that both isozymes of yALDH are present in the sample mixture.

### *Simultaneous Enzyme Inhibition Assay/ESI–MS Analysis of yALDH Reacted with DSF and MeDTC-SO*

The traditional approach to study protein–drug interactions includes such techniques as incorporation of radiolabel, immunofluorescence, and site-directed mutagenesis. Although these methods have proved useful, insight into the mechanism of action of the drug has been somewhat indirect or indeterminant. However, with the recent advances in ESI–MS, it is possible to determine molecular weights of both native proteins and protein–drug adducts using only low picomole-femtomole amounts of material [13]. Furthermore, as demonstrated in this work, by simultaneously conducting an enzyme inhibition assay coupled with ESI–MS analyses, it is possible to readily demonstrate a causal link between the biological and physical measurements. Specifically, the nature yALDH isozymes were initially subjected to both ESI–MS (53,960 and 54,095 Da; ±5 Da) and enzyme activity analysis (~100% activity determined). Subsequently, the yALDH was reacted *in vitro* independently with DSF, MeDDC, and MeDTC-SO. All these reacted protein samples were analyzed simultaneously by enzyme inhibition assay (Fig. 2) and ESI–MS (Fig. 3). Normal (control) yALDH activity was determined in the absence of any inhibitor and measured by determining the formation of NADH at 340 nm. Furthermore, MeDCC was used as a "substrate control" since it has been demonstrated not to inhibit ALDH [14].

### *Inhibition of yALDH by DSF and MeDTC-SO*

DSF. Active, native yALDH protein upon treatment with DSF lost all enzyme activity (Fig. 2). However, treatment with MeDDC resulted in no discernible loss of yALDH activity. Simultaneous ESI–MS analysis revealed no detectable mass shift of the proteins (Fig. 3A), indicating that no intermolecular covalent modification had occurred.

Vallari and Pietruszko [6] originally hypothesized, without direct physical evidence, that DSF inhibits ALDH by forming a protein intramolecular cystein bridge. Formation of an intramolecular disulfide bond would results in a reduction of two mass units from the molecular weight of the native protein. This mass difference is less than the detectable change in mass difference (±0.01%) using conventional ESI–MS analysis. However, enzyme inhibition data combined with the ESI–MS analysis strongly indicate that

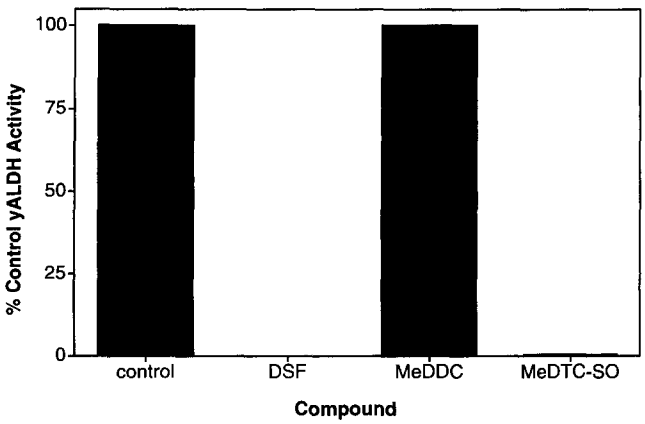


**FIG. 2. Triplicate inhibition studies of enzyme activity for yALDH treated *in vitro* independently with DSF, MeDDC, and MeDTC-SO, performed simultaneously with ESI–MS analysis of resulting products (see Fig. 3). Values are reported as a percent of "control" yALDH activity, which was determined in the absence of any inhibitor. Enzyme activity was determined spectrophotometrically at 340 nm over a 3-min period monitoring the formation of NADH.**

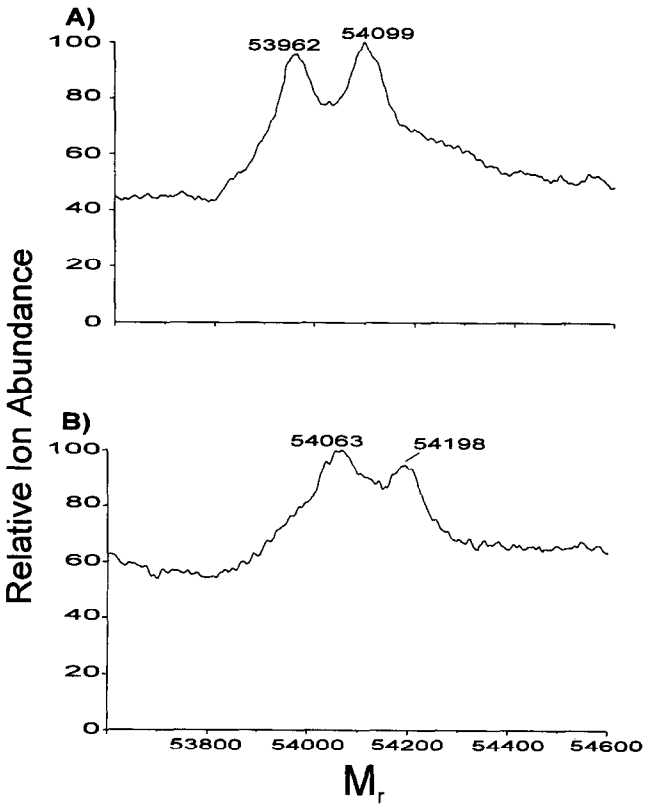


**FIG. 3. ESI–MS analysis of yALDH performed in conjunction with the enzyme inhibition assay (see Fig. 2). (A) Positive ion ESI–MS of native yALDH isozymes, mitochondrial derived (53,962 ± 5 Da) and cytosolic derived (54,099 ± 5 Da). (B) Positive ion ESI–MS of yALDH reacted *in vitro* with MeDTC-SO. In both cases, a mass shift of +~100 Da was observed.**

formation of such an intramolecular bond is occurring when yALDH is reacted *in vitro* with DSF.

MeDTC-SO. yALDH when reacted with MeDTC-SO also lost almost all enzyme activity (Fig. 2). However when the treated protein was analyzed by ESI–MS, a mass shift of +≈100 Da for both isozymes was observed (Fig. 3B). In this case, direct evidence that an intermolecular covalent adduct was responsible for loss of enzyme activity was obtained using the combined bioassay/ESI–MS approach.

### *Recovery of yALDH Activity after Treatment with DTT*

DSF. The presence of an intramolecular disulfide bridge at the active site of yALDH would induce some steric strain and possible change in active site conformation. More importantly, however, it could possibly remove the active site participation of the sulfhydryl group of $Cys_{302}$, shown to be essential for yALDH activity [15, 16]. Reaction of the modified protein with a reducing agent such as DTT should reduce this intramolecular bond and restore enzyme activity. Indeed, addition of 1 mM DTT (pH 7.5) to DSF-inhibited yALDH resulted in ~95% recovery of enzyme activity. A concomitant ESI–MS analysis revealed no discernible mass shift (data not shown) from the molecular weight determined for inhibited yALDH (shown in Fig. 3A).

MeDTC-SO. Treatment of MeDTC-SO-inhibited yALDH with DTT at pH 7.5 resulted in no recovery of enzyme activity nor any change in molecular weight (data not shown). However, addition of 1 mM DTT at pH 9.0 resulted in almost 100% recovery of enzyme activity and a mass loss of ~100 Da as observed by ESI–MS (Fig. 4). This latter result indicates the loss of the adduct to afford the native enzyme.

### *Mechanisms of Inhibition of yALDH*

Based on the simultaneous enzyme inhibition assay and ESI–MS analysis, two distinct mechanisms of yALDH inhibition are apparent. In the case of the parent drug DSF, it appears to act by oxidizing proximal cysteines, known to reside in the active site [15]. The formation of the disulfide bridge would inhibit enzyme activity either by inducing conformational change at the active site or removing the -SH group of $Cys_{302}$. Enzyme activity is readily recovered upon treatment with DTT, which reduces the intramolecular disulfide bridge, and this is all summarized in Fig. 5. It is interesting to note that based on these data, numerous sterically suitable compounds containing a disulfide bridge may possibly be able to inhibit ALDH activity.

The interaction of MeDTC-SO with yALDH results in inhibition of the enzyme by formation of a stable covalent

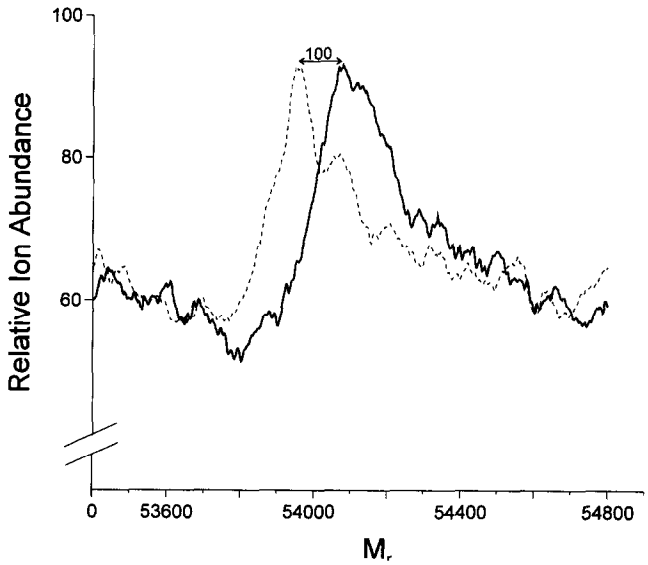


**FIG. 4. Effect of DTT on MeDTC-SO-inhibited yALDH as determined by ESI–MS analysis. Native yALDH (cytosolic and mitochondrial) were treated with MeDTC-SO and shown to be enzymatically inactive as well as shifted by +~100 Da. This is shown by the thick bold line in the figure. The protein adduct was treated with 1 mM DDT at pH 9.0, was reanalyzed subsequently by ESI–MS, and is shown by the dotted line. This resulted in a mass loss of ~100 Da from adducted proteins.**

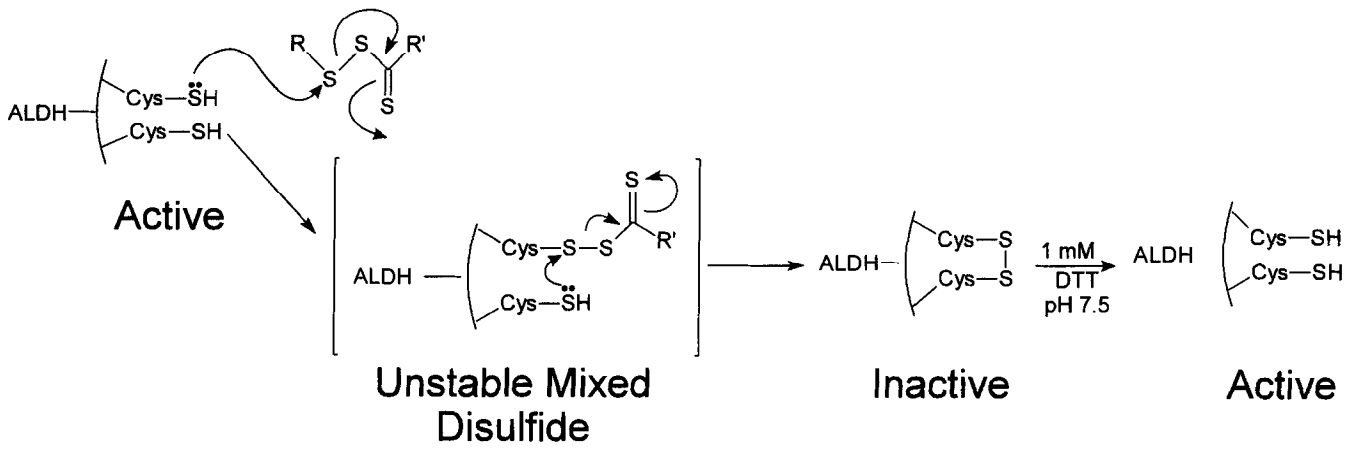


**FIG. 5. Proposed mechanism of yALDH inhibition after *in vitro* treatment of the enzyme with DSF, as well as recovery of enzyme activity after treatment with DTT at pH 7.5.**

adduct. The increase in molecular mass of the adducted protein by 100 Da strongly indicates the formation of a carbamoyl adduct (Fig. 6). This could result from nucleophilic attack by an essential sulfhydryl group of the protein on the electrophilic center of the carbonyl carbon of MeDTC-SO. This type of reaction is consistent with a recent finding reported by Jin *et al.* [17] who structurally characterized *in vivo*-derived glutathione conjugates isolated from rats treated with DSF. They identified *S*-methyl *N*,*N*-diethylthiocarbamoyl glutathione conjugates that were purported to be formed by reaction of MeDTC-SO with glutathione. Furthermore, it is likely that the site of

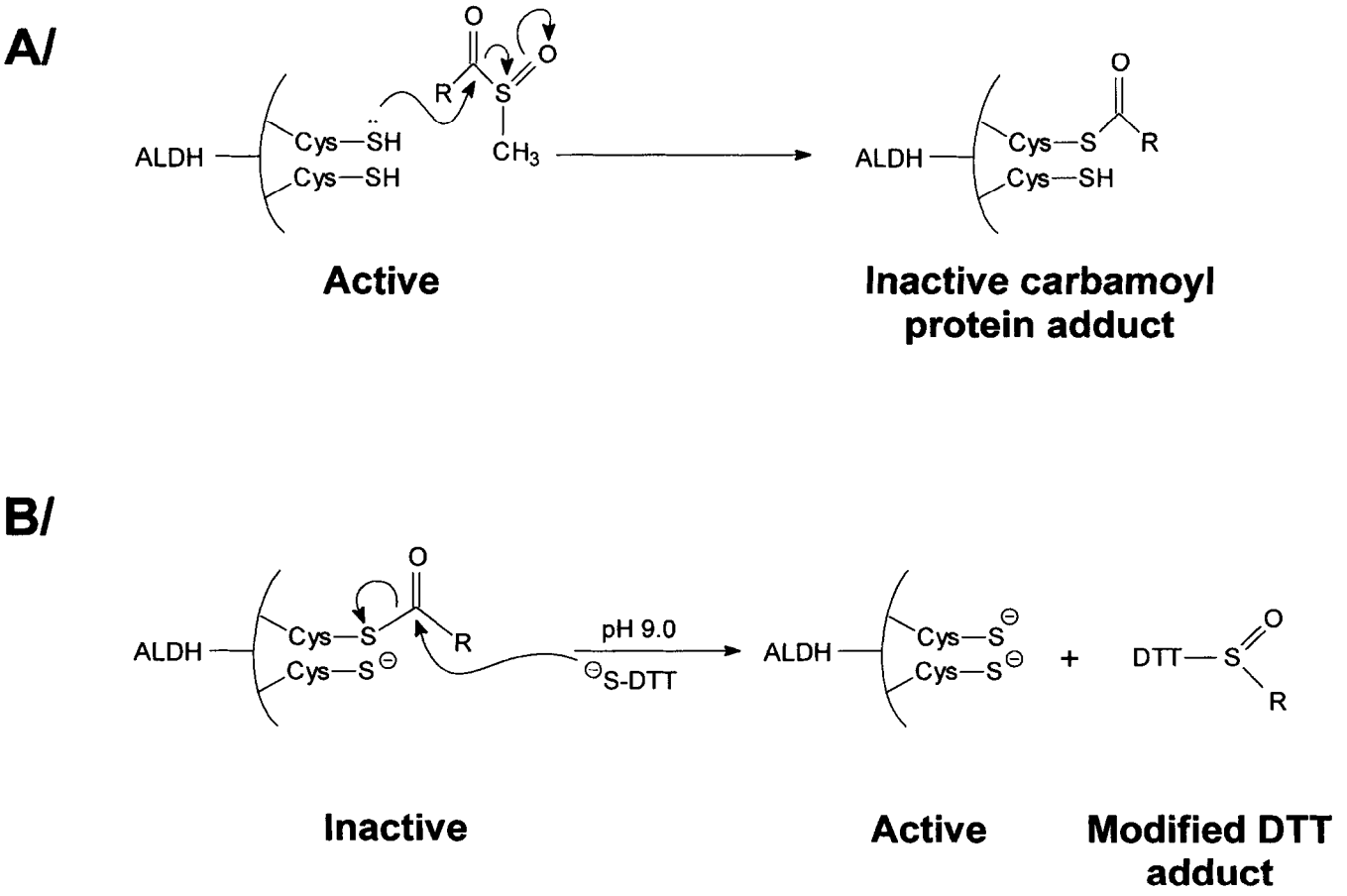


**FIG. 6. (A) Proposed mechanism of yALDH inhibition after *in vitro* treatment with MeDTC-SO. (B) Subsequent recovery of activity after treatment of the inactive enzyme with DTT at pH 9.0.**

adduction is either $Cys_{302}$, shown to be essential for yALDH activity [15, 16] or a proximal site which sterically hinders the $Cys_{302}$ sulfhydryl group. This mechanism of inhibition has also been proposed previously by Wiseman and Abeles [18] to explain the inhibition of yALDH by a metabolite of the mushroom toxine, coprine.

Treatment of MeDTC-SO-inhibited yALDH with 1 mM DTT at pH 9.0 resulted in recovery of enzyme activity and loss of 100 Da corresponding to the carbamoyl adduct. This is due to the increased nucleophilic character of DTT at high pH ($pK_a$ DTT ~8.5), thus facilitating nucleophilic attack by the anionic sulfur of DTT on the carbonyl group of the thioester adduct (see Fig. 6B). This results in acyl cleavage of the thioester adduct to affort native, as well as active yALDH.

Previous studies have shown that reducing agents such as DTT and 2-mercaptoethanol are not able to affect ALDH enzymes that are inhibited *in vivo* [6, 19]. Yet another study reported the inability of 10 mM DTT to recover hepatic ALDH activity *in vitro* after DSF administration *in vivo;* however, the incubations were done at pH 7.4 [20]. The data presented here demonstrate that, consistent with earlier reports, the covalent adduct is stable in the range of physiologically buffered pH (pH 7.5). At elevated pH (pH 9), although the adduct is stable to alkali alone, it is no longer stable to treatment with DTT. Treatment of adducted yALDH with DTT at elevated pH not only released the adduct as indicated by an ~~100 Da shift, but importantly, yALDH activity was also recovered. Furthermore, our proposed mechanism is consistent with previous radiolabeling results employing [$^{14}$C]DSF [6], [$^{14}$C]DDC [6], [$^{35}$S]DSF [20], and $^{14}$C-labeled piperidinocarbanoylthiomethylester analogue of DDC [21].

In conclusion, based on these studies, at least two different mechanisms of ALDH inhibition by DSF and related biotransformation products are possible. The *in vitro* inhibition of yALDH by DSF may be due to oxidation of proximal cystines at the active site. However, since DSF is rapidly reduced to DDC *in vivo,* it is unlikely that this mechanism is of any physiological significance unless new disulfide containing metabolites are subsequently produced. The combined approach of enzyme inhibition assay coupled with ESI–MS analysis reveals that inhibition of yALDH by MeDTC-SO is due to a protein–metabolite adduct formation. Moreover, while DTT is able to restore activity of ALDH inhibited by DSF *in vitro,* it has little effect on inhibition by MeDTC-SO in the physiological pH range. Only at elevated pH (pH 9) was DTT able to recover yALDH activity and release the ~100 Da adduct.

The use of modern MS analysis of protein–adduct complexes is still in its infancy. However, it is evident that the combined approach of specific bioassay coupled with MS analysis of the intact protein offers a powerful approach for mechanistic studies between drugs/toxins and their protein targets. Our study of the inhibition of yALDH by DSF and metabolites serves as a model approach for these types of analyses. Armed with this technique it is possible to conduct a systematic study combining the direct analysis of the protein and protein–drug interaction with bioassay. This approach links information obtained from bioassay to a direct physical measurement of accurate molecular weight determination from ESI–MS. This approach requires very little sample handling and manipulation and should prove useful to investigate the mechanisms by which adducts alter many other intact proteins.

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