Biochemical Pharmacology, Vol. 60, pp. 947–953, 2000.
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ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00435-4

Role of Disulfiram in the *In Vitro* Inhibition of Rat Liver Mitochondrial Aldehyde Dehydrogenase

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ABSTRACT. The alcohol aversion therapy drug disulfiram has been shown to inhibit hepatic aldehyde dehydrogenase (ALDH), one of the key enzymes involved in ethanol metabolism. It is believed by some that disulfiram could be one of the active inhibitors *in vivo*. However, the actual interaction between disulfiram and ALDH remains ambiguous. We report here that when disulfiram inhibited recombinant rat liver mitochondrial ALDH (rlmALDH) *in vitro*, no significant molecular mass increase was detected during the first 30 min as determined by on-line HPLC–electrospray ionization mass spectrometry (LC–MS). This indicated that the inhibition *in vitro* was not caused directly by covalent adduct formation on the enzyme. We subsequently subjected both control and disulfiram-inhibited rlmALDH to Glu-C proteolytic digestion. LC–MS analysis of the Glu-C digestion of disulfiram-inhibited enzyme revealed that one peptide of M_r = 4821, which contained the putative active site of the enzyme, exhibited a mass decrease of 2 amu as compared with the same peptide found in the Glu-C digestion of the control (M_r = 4823). We believe that the loss of 2 amu indicated that inhibition of rlmALDH *in vitro* was due to formation of an intramolecular disulfide bond between two of the three adjacent cysteines in the active site, possibly via a very rapid and unstable mixed disulfide interchange reaction. Further confirmation of the intramolecular disulfide bond formation came from the fact that by adding dithiothreitol (DTT) we were able to recover partial enzyme activity. In addition, the peptide of M_r = 4821 observed in the Glu-C digestion of the disulfiram-treated ALDH reverted to M_r = 4823 after treatment with DTT, which indicated that the disulfide bond was reduced. We, thereby, conclude that disulfiram inhibited rlmALDH by forming an intramolecular disulfide, possibly via a fast intermolecular disulfiram interchange reaction. BIOCHEM PHARMACOL **60**;7:947–953, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. aldehyde dehydrogenase; disulfiram; enzyme inhibition; mass spectrometry; protein–drug interaction; adduct structure; thiol oxidation

Disulfiram (Antabuse) has been used clinically to treat recovering alcoholics for over 50 years. It exerts its pharmacologic effect by irreversibly inhibiting hepatic ALDH,** one of the key enzymes involved in alcohol metabolism. Inhibition of ALDH gives rise to the accumulation of acetaldehyde upon alcohol ingestion. The elevated levels of acetaldehyde lead to a series of unpleasant physical responses characterized by nausea, vomiting, tachycardia, and hypotension [1].

Although the metabolism of disulfiram is well described, efforts are still being made to identify and characterize the ultimate *in vivo* metabolite responsible for the inhibition of ALDH. The parent drug, disulfiram, has been shown to be an inhibitor of ALDH both *in vitro* and *in vivo* [2, 3]. Although it has been found to be reduced rapidly and completely *in vivo* to its monomer, DDC [4], it has been proposed that a small amount of DDC can be reoxidized to form disulfiram intracellularly [3, 5–10]. Hence, the parent drug, disulfiram, has also been suggested to be the active enzyme inhibitor *in vivo* [3, 5, 6, 8, 10]. Subsequently, it was shown that treatment of rats with cytochrome P450 inhibitors prior to disulfiram administration blocked the inhibition of ALDH *in vivo*. This finding suggests that a metabolite of disulfiram is responsible for the inhibition of ALDH [11]. One of the metabolites, MeDTC-SO, has been detected and identified in rat plasma after drug administration [12–14]. In addition, it has been found to be a potent inhibitor of ALDH *in vitro* and *in vivo* [12, 15–20], and, thus, MeDTC-SO currently is believed by some to be the active inhibitor *in vivo*.

Since the possibility of disulfiram being the active inhibitor *in vivo* cannot be ruled out absolutely, efforts have been made to characterize its interaction with ALDH *in vitro*. Disulfiram has been reported to react with sulfhydryl

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** *Abbreviations:* ALDH, aldehyde dehydrogenase; rlmALDH, recombinant rat liver mitochondrial aldehyde dehydrogenase; LC–MS, on-line HPLC-electrospray ionization mass spectrometry; DDC, diethyldithiocarbamate; DTT, dithiothreitol; MeDTC-SO, *S*-methyl-*N, N*-diethylthiocarbamoyl sulfoxide; and *p*HAP, *p*-hydroxyacetophenone.

Received 16 February 2000; accepted 28 March 2000.

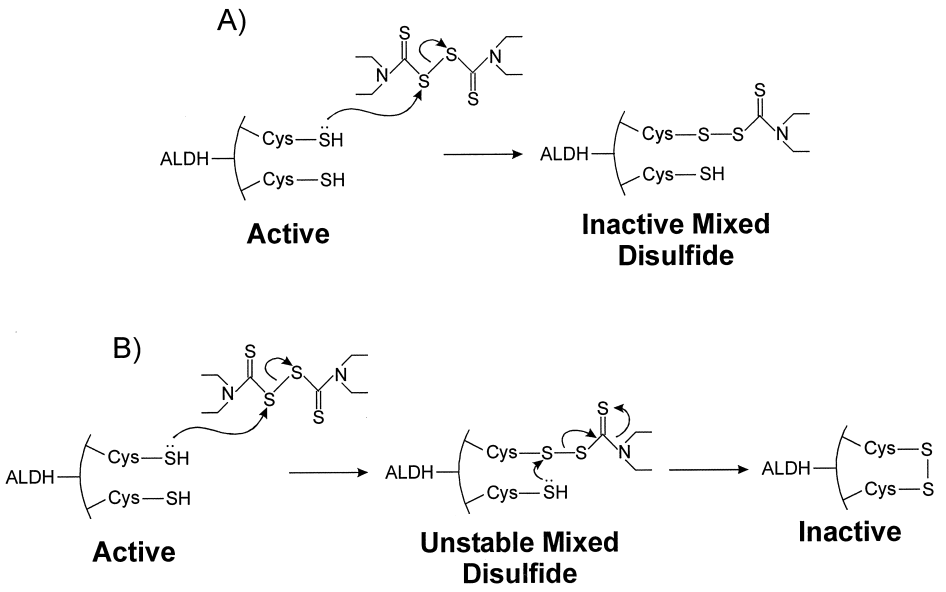


FIG. 1. Possible mechanisms of interaction between disulfiram and ALDH. (A) Inhibition of ALDH via the formation of a stable intermolecular mixed disulfide between disulfiram and an active site thiol. (B) Inhibition of ALDH caused by the formation of an intramolecular disulfide bond between the active site thiol and the thiol of another cysteine residue via an unstable mixed disulfide adduct.

groups on various proteins by forming intermolecular mixed disulfides [21–23]. The early reports by Kitson [6] showed that disulfiram causes an initial partial inhibition of ALDH followed by a gradual irreversible loss of activity. He thus proposed that disulfiram inhibits ALDH *in vitro* by forming an intermolecular mixed disulfide, presumably with the active site thiol [6] as illustrated in Fig. 1A. Subsequently, Vallari and Pietruszko [24] demonstrated that ALDH was inhibited by [^{14}C]disulfiram without incorporation of radiolabel and that 34 free sulfhydryls in each ALDH tetramer decreased to 30 per tetramer. However, Kitson [25] reported that radiolabel incorporation could be detected within the first few minutes of mixing when ALDH was inhibited by [^{14}C]disulfiram. However, the bound radioactivity disappeared over a period of 1 hr. As a result, they both hypothesized that ALDH inhibition by disulfiram could be caused by the formation of an intramolecular disulfide bond between the active site thiol and the thiol of another cysteine residue, potentially via a mixed disulfide interchange reaction as shown in Fig. 1B. This hypothesis was confirmed further by our findings that disulfiram inhibits yeast ALDH without any significant change in the detected molecular mass of the enzyme [17]. In addition, Sanny and Weiner [26] reported that [^{35}S]disulfiram was found to be bound to inhibited ALDH, yet it did not affect the pre-steady-state burst of the enzyme. Their interpretation of the result was that disulfiram inhibited ALDH by forming an intermolecular mixed disulfide with a thiol located outside of the active site [26].

Since there is some significant ambiguity associated with the mechanism of interaction between disulfiram and ALDH, we investigated the reaction between disulfiram and rlmALDH *in vitro* using LC–MS. We demonstrated that disulfiram inhibits rlmALDH initially without significantly changing the apparent molecular mass of the protein, while covalent adducts were detected after 1 hr. LC–MS analysis of the Glu-C digestions of disulfiram-inhibited rlmALDH revealed that the peptide fragment spanning the region of the active site of rlmALDH showed a molecular mass loss of 2 amu. This decrease of 2 amu was indicative of an intramolecular disulfide bond formation between two of the three adjacent cysteines within the active site of rlmALDH.

MATERIALS AND METHODS

Materials

Disulfiram was recrystallized twice in ethanol before use. NAD^+ (grade 1 free acid, 100%), DTT, and Glu-C were purchased from Boehringer Mannheim. Acetaldehyde, β-mercaptoethanol, and epoxy-activated Sepharose 6B were obtained from the Sigma Chemical Co. The recombinant rat liver mitochondrial ALDH cDNA in pT7–7 was a gift from Dr. Henry Weiner (Purdue University).

Expression of rlmALDH in Escherichia coli *and Purification*

The enzyme was isolated using the method of Jeng and Weiner [27] with some modifications. Specifically, *E. coli* cells carrying the plasmid that contains the rlmALDH cDNA were grown in 2 L of Terrific Broth supplemented with 100 μg/mL of ampicillin. Subsequently, the cells were harvested and lysed. The lysate was purified using a *p*HAP affinity column. ALDH was eluted with 10 mM *p*HAP in column equilibration buffer (20 mM sodium phosphate, 1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, pH 7.4). Eluant fractions with a protein concentration of > 0.5 mg/mL were pooled, and dialyzed twice against 1 L of equilibration buffer over 24 hr each.

ALDH Activity Assay

A microtiter-based method was employed as described by Nelson and Lipsky [28] with some modifications. Briefly, 10 μL of purified rlmALDH was dispensed into microtiter plate wells in triplicate, followed by the addition of 190 μL of 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 by monitoring the formation of NADH at 340 nm.

Inhibition of rlmALDH by Disulfiram

Purified rlmALDH was first concentrated to approximately 4 mg/mL by spinning at 6.4 *g* using a Michrom Spin Filter (Chrom Tech). Then 80 μL of concentrated rlmALDH was dialyzed against 200 mL of buffer (20 mM sodium phosphate, 1 mM EDTA, 50 mM NaCl, pH 7.4) at room temperature for 3 hr to remove DTT using a Millipore Drop Filter (Millipore). The dialyzed rlmALDH was incubated at room temperature with disulfiram in methanol at a 1:4 monomeric ratio for 15 min at pH 7.4. The enzyme activity was assayed to measure the extent of inhibition, and the remaining free disulfiram was then removed by a Sephadex gel filtration column (Clontech).

LC–MS of Intact Proteins

Intact rlmALDH was analyzed directly by employing a preconcentration protein trap cartridge on-line with LC–MS using a Finnigan MAT 900 mass spectrometer. The latter was equipped with a Finnigan MAT electrospray source (Finnigan MAT GmbH). Forty microliters of rlmALDH solution (*ca.* 10 μM in 20 mM sodium phosphate, 1 mM EDTA, and 50 mM NaCl, pH 7.4) was preconcentrated onto a 1 × 10 mm reversed phase protein trap (Michrom Bioresources), which replaced the sample loop of the LC injector. Salts and other hydrophilic material were removed by washing the trap with 200 μL of mobile phase A before the protein trap was placed in-line with the column for development of the gradient. LC separation of rlmALDH from minor impurities was performed on a Michrom UMA (Michrom Bioresources) using a PLRP-S reversed phase, 300 Å pore size, 1.0 × 50 mm column (Michrom Bioresources). A gradient consisting of mobile phase A (98:1:1:0.5:0.02 water:acetonitrile:*n*-propanol:acetic acid:trifluoroacetic acid) and mobile phase B (80:10:10:0.5:0.02 acetonitrile:water:*n*-propanol:acetic acid:trifluoroacetic acid) (20–70% mobile phase B) was run for 10 min at a flow rate of 75 μL/min. The entire effluent was directed into the electrospray interface and ionized using an electrospray voltage of 4.08 kV, with a 3 L/min flow of N_2 as the sheath gas. Ions were detected by a PATRIC™ (Position and Time Resolved Ion Counter) scanning array detector with an 8% mass window. The magnet was set to scan over a mass range of m/z 800 to 3000, at a rate of 2 sec/decade. The multiply charged ion series for each protein analysis was transformed using a Finnigan MAT algorithm software package to afford the M_r.

LC-MS of Glu-C Protein Digests

Forty microliters of control and disulfiram-inhibited rlmALDH were combined separately with 80 μL of ammonium acetate buffer (20 mM ammonium acetate, 1% acetic acid, pH 3.7) and 4 μL of a 50 μg/μL solution of Glu-C. The mixture was incubated at 37° for 2 hr. The digestion products were analyzed by LC–MS immediately after 2 hr. LC separations of peptides from Glu-C digestion of either control or disulfiram-inhibited rlmALDH were performed using a Monitor C-18 (Michrom Bioresources), 100 Å pore size, 1.0 × 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 ions were detected by the PATRIC™ detector with a mass window of 4%, while the magnet was set to scan over a range of m/z = 500 to 1800, at a scan rate of 3 sec/decade. The multiply charged ions corresponding to individual Glu-C derived peptides were transformed using the Finnigan MAT algorithm software to afford M_r values.

RESULTS AND DISCUSSION

Disulfiram has been shown to be an irreversible inhibitor of hepatic ALDH [2, 3]. Numerous metabolites have been identified and characterized, among which MeDTC-SO currently is believed by some to be the active metabolite *in vivo*. It has also been proposed previously that disulfiram could be re-formed intracellularly after its rapid reduction to DDC *in vivo* [3, 5–10, 29]. Therefore, disulfiram itself may be playing a possible role in the *in vivo* inhibition of ALDH. As a result, studies have been performed to characterize the interaction between disulfiram and ALDH *in vitro*, yet the details regarding the interaction remain ambiguous. In this study, we characterized the interaction between disulfiram and rlmALDH by first incubating rlmALDH with disulfiram *in vitro*. Both the control and inhibited enzymes were subjected to a series of analyses by LC–MS.

Inhibition of rlmALDH by Disulfiram

rlmALDH was incubated with various concentrations of disulfiram *in vitro* at pH 7.4 for 15 min. The extent of inhibition of ALDH activity was determined (Fig. 2), and the IC_{50} was found to be 36.4 μM. When rlmALDH was incubated with disulfiram at a 1:4 monomeric ratio at room temperature in *p*HAP equilibration buffer without DTT at pH 7.4, the inhibition increased from 57.7 ± 11.7% at 5 min to 83.6 ± 8.6% over a period of 3 hr. After 24 hr, the control rlmALDH showed a decrease of approximately 7% in enzyme activity. However, the disulfiram-treated enzyme was inhibited by virtually 100% (Fig. 3).

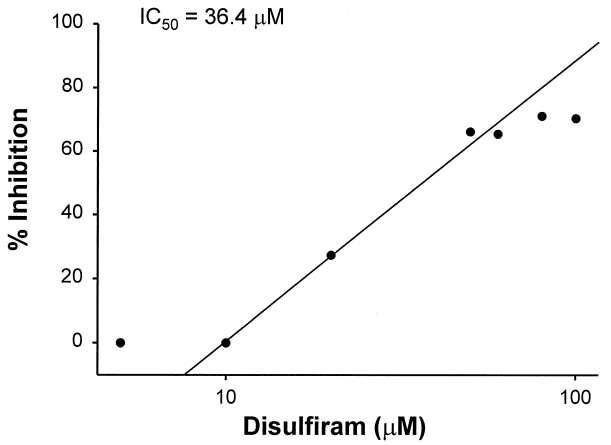


FIG. 2. Inhibition of rlmALDH activity *in vitro* by disulfiram at pH 7.4, with various concentrations of disulfiram. Inhibition of enzyme was determined by measuring NADH formation spectrophotometrically at 340 nm.

LC–MS Analysis of Control and Disulfiram-Inhibited rlmALDH

Initially, control rlmALDH was subjected to LC–MS analysis, and a relative molecular mass (M_r) of 54,366 Da (±0.01%) was determined (Fig. 4A). This molecular mass corresponded well with the theoretical mass of rlmALDH of 54,368 Da based on sequence analysis. Subsequently, disulfiram-inhibited rlmALDH was also subjected to LC–MS analysis. We found that the M_r of the inhibited enzyme remained at 54,364 Da even after a 30-min incubation (Fig. 4B). This mass showed no significant change from that of the control within instrumental mass measurement error (±0.01%), even though the enzyme was inhibited by up to 75% of its activity. The molecular mass of disulfiram-treated rlmALDH remained unchanged up to approximately 1 hr. These findings suggested that initial

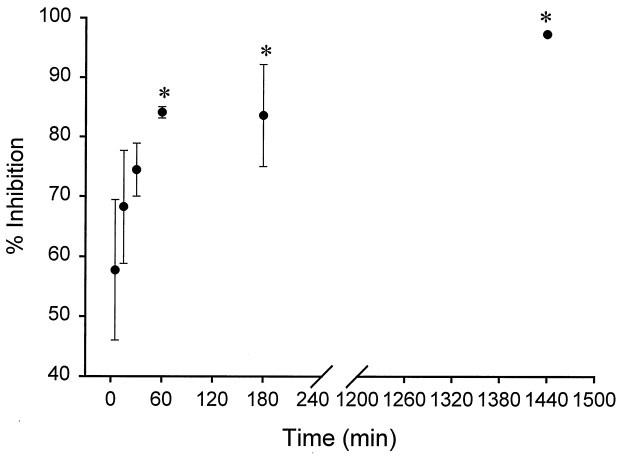


FIG. 3. Extent of *in vitro* rlmALDH inhibition by disulfiram as a function of time. Each time point was carried out at pH 7.4, at room temperature, and enzyme inhibition was determined as described in Fig. 2. Shown is a composite of 5 repeat experiments with SEM. An asterisk (*) denotes detection of covalent adducts on rlmALDH.

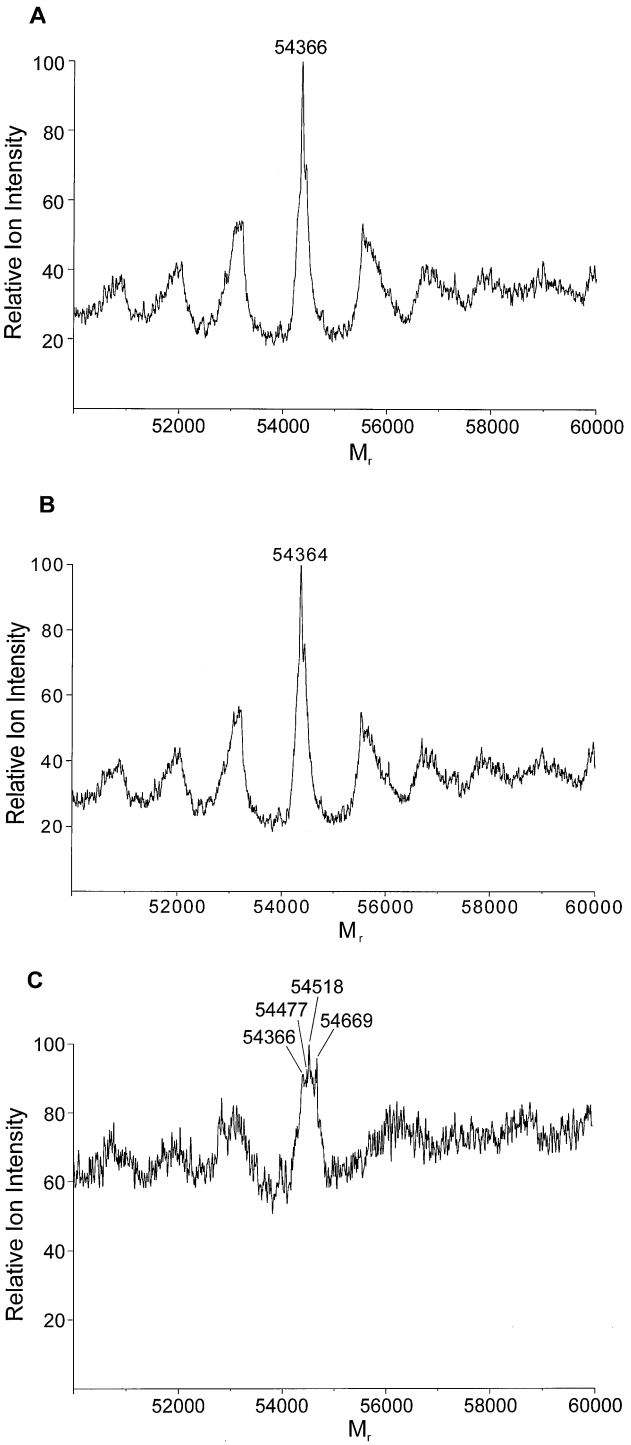
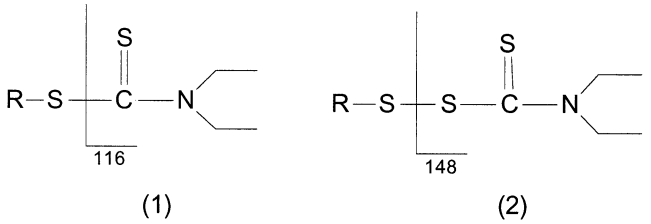


FIG. 4. LC–MS analysis of control and disulfiram-inhibited rlmALDH. (A) Positive ion mass spectrum of control rlmALDH, which showed a M_r of ~54,366 Da (mass error ± 0.01%). (B) Positive ion mass spectrum of disulfiram-inhibited rlmALDH (30-min incubation); the M_r = ~54,364 Da is within experimental error the same as detected in panel A. (C) Positive ion mass spectrum of disulfiram-inhibited rlmALDH (3-hr incubation). The molecular mass of the enzyme was shifted to ~54,477, ~54,518, and ~54,669 Da with some remaining unmodified rlmALDH at ~54,366 Da.

inactivation of rlmALDH by disulfiram *in vitro* was not due to covalent adduct formation on the protein, as previously suggested by Kitson [25].

These results are in agreement with previous reports of Vallari and Pietruszko [24] and Veverka *et al.* [17] that inhibition of human and yeast ALDH, respectively, is caused by the possibility of an intramolecular disulfide bridge formation via a mixed disulfide interchange reaction. On the other hand, Kitson demonstrated that radiolabel incorporation disappears over 1 hr, suggesting that the *in vitro* inactivation of sheep liver cytosolic ALDH by disulfiram could be caused eventually by an intramolecular disulfide. He also was able to detect the initial fast formation of a relatively stable mixed diethyldithiocarbamoyl adduct, as indicated by extensive radiolabel incorporation, up to 30 min prior to its conversion to the internal disulfide bond [25]. However, in our study disulfiram-inhibited rlmADLH was subjected to LC–MS analysis directly following incubation, and the shortest incubation time was approximately 10 min. We did not detect the presence of any covalent adducts on the inactivated enzyme as reflected by a change in the molecular mass. As a result, we propose that any potential mixed disulfide interchange reaction took place in our system much more rapidly than what Kitson had reported.

However, upon incubating rlmALDH with disulfiram for 3 hr, significant adducts were detected on rlmALDH characterized by the mass increases of ~111, ~152, and ~303 Da, and, thereby, the M_r of rlmALDH was shifted to ~54,477, 54,518, and 54,669 Da. There was also still some unmodified rlmALDH detected at ~54,366 Da (Fig. 4C). The molecular mass increases of ~111 and ~152 Da were consistent with formation of a diethylthiocarbamoyl (1) and diethyldithiocarbamoyl (2) adduct, respectively, as shown below. Formation of (1) occurs via nucleophilic attack of a protein Cys sulfhydryl group on the thiocarbamoyl carbon of disulfiram. The formation of (2) occurs via nucleophilic attack of the Cys sulfhydryl group on disulfiram to form a mixed disulfide adduct. Finally, the addition of ~303 Da corresponds to the formation of two diethyldithiocarbamoyl adducts on the intact rlmALDH, within the experimental mass measurement error of ±0.01%. We also observed that after 24 hr, the predominant adduct contained the two diethyldithiocarbamoyl adducts, affording a M_r of ~54,665 Da (data not shown).



We have shown that rlmALDH inhibition increased up to 75% in the first 30 min without any detectable molecular mass increases (Fig. 3). In addition, disulfiram has been reported by other investigators to be reactive towards thiols on proteins and has been used as a thiol titrating agent [22, 23]. We, therefore, propose that these adducts found after a long period of incubation could be due to nonspecific mixed disulfide formation with nonessential thiols on rlmALDH, although it is possible that these adducts also contributed to further loss of enzyme activity, which is being investigated further.

LC–MS Analysis of Glu-C Digestions

Glu-C digestions were performed on both the control and disulfiram-inhibited rlmALDH following a 15-min incubation and removal of remaining disulfiram. Glu-C is a proteolytic enzyme that cleaves at the C-terminal side of Glu amino acid residues. It affords specific, defined proteolytic peptides that are directly amenable to LC–MS analysis. The overall spectrum of the Glu-C digestion of the control enzyme looked very similar to that of the disulfiram-inhibited enzyme. Upon careful inspection of the spectra, we observed one ion at $MH^+ = 4823$ in the control Glu-C digestion (Fig. 5A), which was shifted to $MH^+ = 4821$ in the Glu-C digestion of the disulfiram-inhibited rlmALDH (Fig. 5B), and this phenomenon was reproducible in three independent sets of experiments. This ion could be tentatively assigned to the peptide LGCKSPNIIMSDADMDWAVEQAHFALFFNQGQC$_{301}$C$_{302}$C$_{303}$AGSRTFVQE, which contains the three essential adjacent cysteines believed to be part of the active site of ALDH [30–34]. The decrease of 2 amu in this peptide was a further confirmation of the intramolecular disulfide formation, and the intramolecular disulfide bond could be formed between two of the three cysteines.

Effect of DTT on Disulfiram Inhibition

DTT was added to both the control rlmALDH and the disulfiram-inhibited rlmALDH at pH 7.4 to a final concentration of 1 mM. ALDH activity was recovered partially in disulfiram-inhibited rlmALDH, as shown in Fig. 6. However, the recovery did not exceed ~45% of the enzyme activity detected in the control. This was probably due to the relatively low concentration of DTT that we employed. Vallari and Pietruszko [24] showed that a concentration of 20 mM 2-mercaptoethanol was used to facilitate full recovery of enzyme activity after disulfiram treatment *in vitro*.

When DTT was added to the Glu-C digestion of the disulfiram-inhibited rlmALDH, the peptide at $M_r = 4821$ detected previously reverted to $M_r = 4823$ (Fig. 5C). The addition of DTT to the control Glu-C digestion did not change the m/z of the peptide at $M_r = 4823$ (data not shown). The recovery of 2 amu after DTT treatment suggested that reduction of the intramolecular disulfide bond had occurred, which was also an indication of intramolecular disulfide formation upon enzyme inhibition.

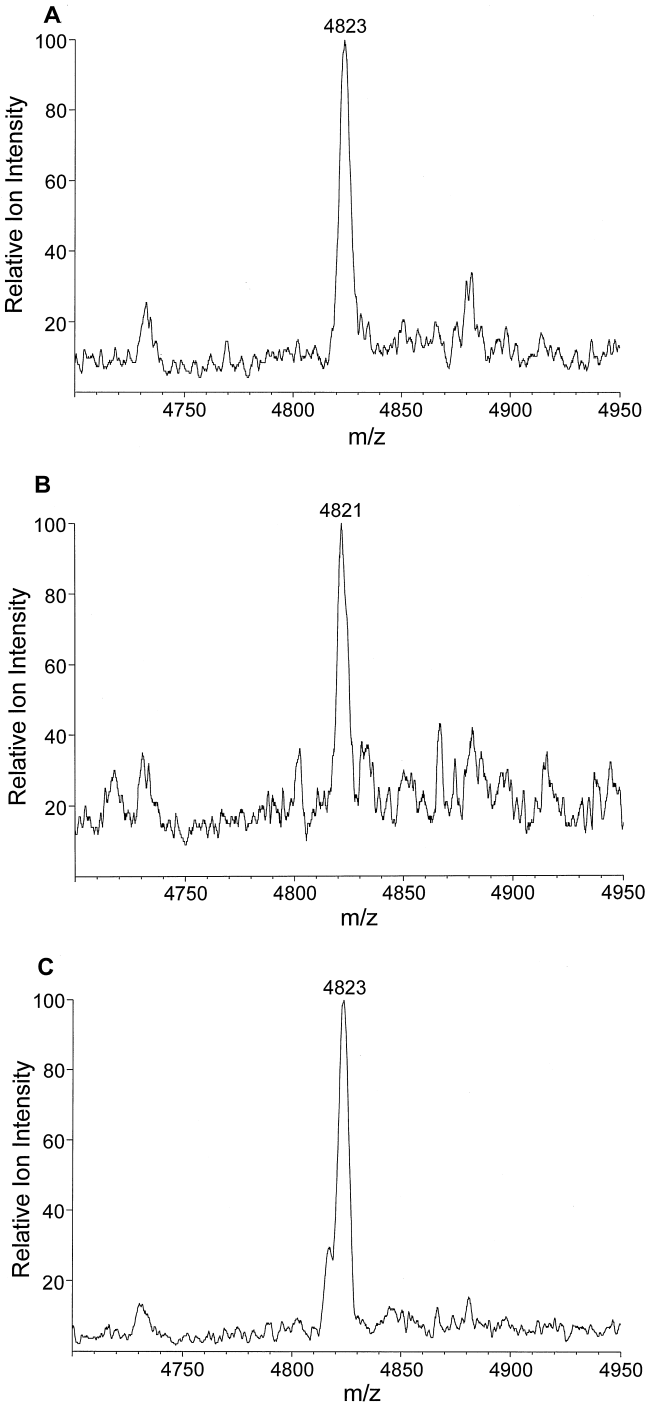


FIG. 5. LC–MS analysis of control and disulfiram-inhibited rlmALDH after Glu-C digestion. (A) LC–MS of Glu-C digestion of control rlmALDH. Partial MS transformed spectrum from *m/z* 4700–4950 of the ion chromatogram signal at an LC retention time of 21.05 min. (B) LC–MS of Glu-C digestion of disulfiram-inhibited rlmALDH. Same conditions as in Fig. 5A. (C) LC–MS of Glu-C digestion of disulfiram-inhibited rlmALDH after reaction with DTT. Same conditions as in Fig. 5A.

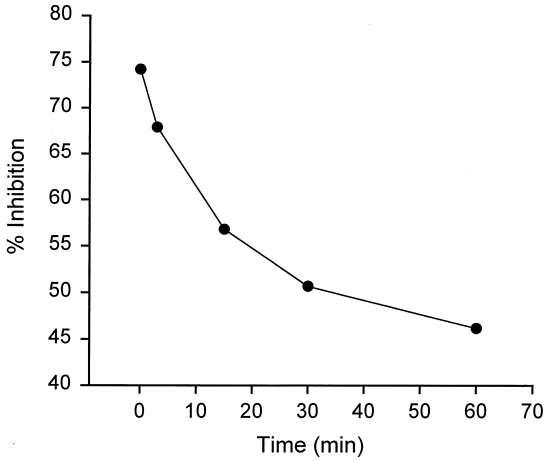


FIG. 6. Recovery of activity in disulfiram-inhibited rlmALDH after DTT treatment. rlmALDH activity was monitored over time after reacting inhibited enzyme with 1 mM DTT at pH 7.4, room temperature. Enzyme activity was determined as described in Fig. 2.

CONCLUSIONS

In conclusion, we have shown that disulfiram rapidly inhibits rlmALDH *in vitro*, by forming an intramolecular disulfide bridge. The formation of this intramolecular disulfide bond involves two of the three adjacent Cys amino acids located at the active site of the enzyme. Prolonged incubation (>1 hr) of rlmALDH with disulfiram does ultimately result in the formation of diethylthiocarbamoyl and mixed disulfide diethyldithiocarbamoyl protein adducts. These adducts may represent nonspecific adduct formation and are unlikely to have any real physiological significance.

Kitson [25] has reported that initial formation of the mixed disulfide adduct is essential for sheep ALDH inactivation and that the conversion to an intramolecular disulfide bridge is slow. Our data indicate that disulfide bridge formation occurs somewhat rapidly. While we cannot rule out that this occurs via an unstable mixed disulfide, we did not detect such an entity in our initial mass measurements of rlmALDH incubated with disulfiram. However, we were able to detect mixed disulfide adducts after incubation of rlmALDH with disulfiram for >1 hr. However, it is important to point out that Kitson [25] used sheep liver cytosolic ALDH for his mechanistic studies. Therefore, the enzyme he studied may well exhibit a different mechanism of interaction with disulfiram *in vitro* as compared with the rat liver mitochondrial enzyme studied in this investigation. Finally, we conclude from this study that disulfiram inhibited recombinant rlmALDH by forming a stable intramolecular disulfide between two of the three adjacent cysteines in the active site.

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.

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