Human mitochondrial aldehyde dehydrogenase inhibition by diethyldithiocarbamic acid methanethiol mixed disulfide: a derivative of disulfiram

Alexander D. MacKerell, jr, Robert C. Vallari and Regina Pietruszko

Center of Alcohol Studies, Rutgers University, New Brunswick, NJ 08903, USA

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A derivative and possible physiological metabolite of disulfiram, diethyldithiocarbamic acid methanethiol mixed disulfide, is shown here for the first time to inactivate the mitochondrial low-K_m isozyme of human aldehyde dehydrogenase (EC 1.2.1.3). By comparing inactivating effects of diethyldithiocarbamic acid mixed disulfides with thiols of increasing chain length evidence is provided that steric hindrance is the reason for lack of inhibition of the mitochondrial enzyme by disulfiram in vitro. Since methanethiol is a normal metabolite [(1983) Annu. Rev. Biochem. 52, 187–222] the results also suggest a mechanism by which aldehyde dehydrogenase is inhibited by disulfiram and diethyldithiocarbamic acid in vivo.

Mitochondrial aldehyde dehydrogenase *Disulfiram* *Diethyldithiocarbamic acid methanethiol mixed disulfide*

1. INTRODUCTION

Disulfiram (tetraethylthiuram disulfide, Antabuse, see fig.1) is an aversive agent used clinically in the treatment of alcoholism. Disulfiram and its primary metabolite diethyldithiocarbamic acid appear to function in vivo by irreversible [1] inhibition of both cytoplasmic and mitochondrial low-K_m aldehyde dehydrogenase [2,3]. However, when the low-K_m isozymes were purified from several mammalian species [4–6] it was found that diethyldithiocarbamic acid was non-inhibitory and that disulfiram inhibited only the cytoplasmic isozyme. Recently the mechanism by which disulfiram inhibits the human cytoplasmic isozyme has been reported in detail [7].

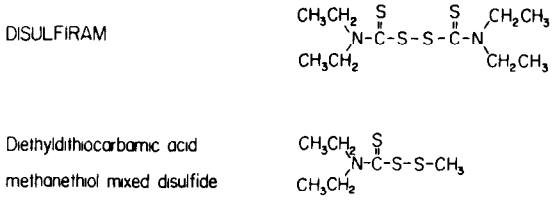

Fig.1. Chemical formulae of disulfiram and diethyldithiocarbamic acid methanethiol mixed disulfide.

Studies indicate that acetaldehyde oxidation in vivo is catalyzed by the mitochondrial isozyme [8]. Due to the ineffectiveness of disulfiram and diethyldithiocarbamic acid with the mitochondrial isozyme in vitro it has been suggested that a metabolite may inhibit in vivo. Known metabolic products (carbon disulfide, diethylamine and diethyldithiocarbamic acid methyl ester) [9–11] have been tested but found to be non-inhibitory and experiments using mixed disulfides between diethyldithiocarbamic acid and physiological sulfhydryl compounds (glutathione, cysteine) have been inconclusive [12]. (We synthesized mixed disulfides of diethyldithiocarbamic acid with the following compounds: glutathione, cysteine, coenzyme A, ergothionine, and lipoamide and tested them as inhibitors of the mitochondrial aldehyde dehydrogenase. Only the lipoamide derivative was slightly inhibitory and only when used in a large excess in an extended incubation. Diethyldithiocarbamic acid methyl ester was also tested and found to be ineffective.) Here we examine inhibitory properties

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of a series of diethyldithiocarbamic acid mixed disulfides and suggest an explanation as to why disulfiram and diethyldithiocarbamic acid are potent inhibitors of the mitochondrial isozyme in vivo but not in vitro.

2. MATERIALS AND METHODS

Chemicals used include: glutathione, ergothionine, 2-mercaptoethanol, coenzyme A, lipoamide, EDTA, cysteine, sodium borohydride, silica gel (Sigma); dimethyl disulfide and ethanethiol–octanethiol (Aldrich); thin-layer chromatographic plates (Macherey-Nagel); disulfiram (Ayerst) and nicotinamide adenine dinucleotide (NAD^+) (Boehringer-Mannheim). Synthesis of the various diethyldithiocarbamic acid mixed disulfides (table 1) was accomplished by reacting the appropriate sulfhydryl compound with an equimolar amount of disulfiram in 95% ethanol and stirring the mixture at room temperature for several days. Methanethiol was first formed by addition of sodium borohydride (1/2 equimolar amount) to dimethyl disulfide prior to addition of disulfiram. Analysis and purification of the mixed disulfides were performed using chromatography on silica gel in a hexane:methylene chloride (6:4) system. Overall yields were around 20%. Identification of compounds was performed using ^1H and ^{13}C NMR (see table 1).

The mitochondrial low-K_m E2 isozyme was purified to homogeneity from human liver [13]; prior to use the enzyme was dialyzed against 8 changes of N_2-saturated 30 mM phosphate buffer (pH 6.0) containing 1 mM EDTA at 4°C. Measurement of activity was done spectrophotometrically at 340 nm and 25°C in 30 mM phosphate (pH 7.0) containing 500 μM NAD^+, 1 mM propional-

Table 1

Spectral properties for various diethyldithiocarbamic acid mixed disulfides and their second-order rate constants for inactivation of the mitochondrial (E2) isozyme

Compound	Proton NMR (CCl_4), δ(ppm)	Carbon 13 ($CDCl_3$), δ(ppm) (fully decoupled)	Second-order rate constants $(\mu M \cdot min)^{-1}$
Disulfiram	1.42 (t,12H), 4.01 (q,8H)	11.24(2C), 13.71(2C), 47.37(2C), 51.72(2C), 192.30(2C)	0.001
DDC-methanethiol	1.30(t,6H), 2.46(s,3H), 3.6-4.3(s,4H)	10.27, 11.94, 21.57, 45.72, 50.04, 193.07	20.6
DDC-ethanethiol	1.32(t,9H), 2.86(q,2H), 3.6-4.2(s,4H)	10.98, 12.60, 13.19, 31.82, 46.51, 51.01, 195.22	1.07
DDC-*n*-propanethiol	0.82-2.0(m,11H), 2.83(t,2H), 3.6-4.3(s,4H)	10.80, 12.48(2C), 21.15, 39.77, 46.31, 50.81, 194.92	0.57
DDC-*n*-butanethiol	0.70-2.0(m,13H), 2.83(t,2H), 3.6-4.2(s,4H)	10.76, 12.40, 12.93, 20.93, 29.84, 37.52, 46.26, 50.71, 194.87	0.36
DDC-*n*-pentanethiol	0.70-1.95(m,15H), 2.84(t,2H), 3.6-4.3(s,4H)	10.82, 12.40, 13.24, 21.54, 27.51, 29.96, 37.84, 46.30, 50.78, 195.00	0.18
DDC-*n*-hexanethiol	0.65-1.98(m,17H), 2.84(t,2H), 3.6-4.3(s,4H)	10.89, 12.45, 13.41, 21.86, 27.59, 27.86, 30.76, 37.97, 46.35, 50.87, 195.12	0.005
DDC-*n*-heptanethiol	0.65-1.98(m,19H), 2.85(t,2H), 3.6-4.3(s,4H)	10.94, 12.53, 13.47, 21.97, 27.83(2C), 28.24, 31.09, 38.00, 46.46, 50.93, 195.18	0.001
DDC-*n*-octanethiol	0.65-1.95(m,21H), 2.85(t,2H), 3.6-4.3(s,4H)	10.86, 12.50, 13.47, 22.00, 27.89(2C), 28.49(2C), 31.15, 37.94, 46.37, 50.88, 195.10	0.001
DDC-2-mercaptoethanol	1.30(t,6H), 2.93(t,2H), 3.4-4.3(m,7H)	10.50, 12.20, 41.54, 46.43, 51.15, 57.73, 195.17	0.78

DDC, diethyldithiocarbamic acid. Infrared spectra of DDC-methanethiol revealed the following peaks (V_{max} cm^{-1}); 1483 (N-C=S), 1360 and 1383 (C=S) and 1205, 1155 and 1090 (tertiary amine)

dehyde, and 1 mM EDTA. Protein concentrations were determined by absorption at 280 nm [14]. Enzyme inactivation was determined by adding enzyme to a cuvette containing 30 mM phosphate (pH 7), 1 mM EDTA, 150 μM acetaldehyde, 500 μM NAD^+ and the appropriate mixed disulfide inhibitor in 3 ml total volume. The resulting steady-state rates were then determined spectrophotometrically and compared with control containing no inhibitor. Second-order rate constants were determined using the above system with inhibitors at a stoichiometry of two equivalents (0.46 μM) per enzyme tetramer (0.23 μM), M_r 216000 [14]. Determination of rate constants was performed by measuring the decrease in rate with time and applying the following equation [15]:

$$1/E - 1/E_0 = bK_2t$$

where E represents the active enzyme concentration at time t, E_0 is the active enzyme concentration at $t = 0$, b is the ratio of inhibitor to enzyme and equals 2, K_2 is the second-order rate constant.

3. RESULTS AND DISCUSSION

During this investigation we observed that the human mitochondrial E2 isozyme could be inhibited by disulfiram in the presence of trace amounts of 2-mercaptoethanol. (2-Mercaptoethanol is a sulfhydryl reducing agent which is routinely incorporated into isolation and storage buffers to protect aldehyde dehydrogenase against oxidation and inactivation, but is generally removed from enzyme samples before experimental use. Only following exhaustive dialysis of the mitochondrial isozyme could a consistent and accurate determination of its activity in the presence of disulfiram be obtained.) The diethyldithiocarbamic acid 2-mercaptoethanol mixed disulfide was therefore synthesized (table 1) and tested as an inhibitor of mitochondrial isozyme activity. Addition of an excess of the inhibitor (16 molar equivalents per enzyme tetramer) directly to the reaction already in progress resulted in rapid and total inhibition which was not reversed by dialysis. Subsequent addition of excess 2-mercaptoethanol (10 mM) restored enzyme activity to its original value. Preincubation of the enzyme with stoichiometric amounts of inhibitor (4 molar equivalents per tetramer) prior to assay resulted in almost total loss of initial activity (0–12% of original). These observations are similar to those obtained with the cytoplasmic E1 isozyme using disulfiram as an inhibitor [7].

Although diethyldithiocarbamic acid 2-mercaptoethanol mixed disulfide is a potent inhibitor of the mitochondrial isozyme, 2-mercaptoethanol is not known to occur in living organisms. In search of physiologically occurring compounds which would react with disulfiram or diethyldithiocarbamic acid to yield mixed disulfides (in addition to those listed in section 1), we came upon the recently described transamination pathway for the degradation of methionine [16]. This pathway produces a mercaptan intermediate methanethiol [16–18]. Synthesis and purification of the diethyldithiocarbamic acid methanethiol mixed disulfide (fig.1) was performed (table 1) and its inhibitory properties toward the mitochondrial isozyme examined. Diethyldithiocarbamic acid methanethiol mixed disulfide inhibited the mitochondrial isozyme rapidly (table 1) and at stoichiometric concentrations (fig.2). The inhibition was reversible on addition of 10 mM 2-mercaptoethanol. Dimethyl disulfide (35 mM) did not inhibit the enzyme. The inhibition with diethyldithiocarbamic acid

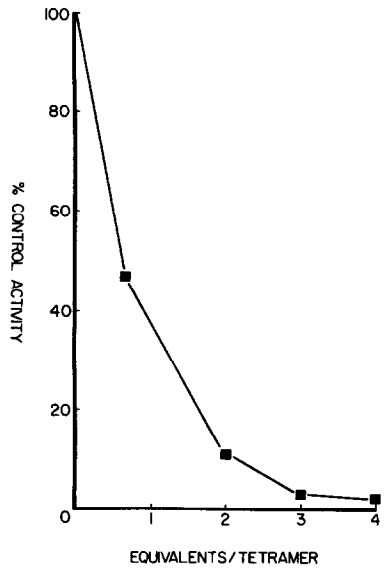

Fig.2. Inactivation of human mitochondrial aldehyde dehydrogenase E2 by diethyldithiocarbamic acid methanethiol mixed disulfide. Inactivation was done as described in text.

methanethiol mixed disulfide occurred in a way analogous to that observed with the 2-mercaptoethanol mixed disulfide and to the inhibition of the cytoplasmic isozyme by disulfiram [7].

Further investigations were designed to determine why compounds such as the diethyldithiocarbamic acid methanethiol mixed disulfide and disulfiram, although structurally similar (fig.1), have different inhibitory properties toward the mitochondrial E2 isozyme but are equally effective with the cytoplasmic E1 isozyme. Since both compounds are neutral, charge was not a consideration; however, size differences indicated the possibility that steric hindrance may play a role in limiting the binding to smaller molecules. This was investigated by measuring the rate constants for the inhibition of the mitochondrial E2 isozyme activity by mixed disulfide compounds of increasing chain length (table 1). It can be seen that diethyldithiocarbamic acid methanethiol mixed disulfide (the smallest molecule in this series) inhibits the mitochondrial isozyme most effectively; as the chain length increases, the rate of inhibition decreases until a degree of inhibition similar to that with disulfiram is obtained. It appears that a similar disulfiram recognition site exists in both cytoplasmic and mitochondrial isozymes but due to a more compact three-dimensional structure disulfiram is precluded from binding in the mitochondrial isozyme in the way necessary to efficiently inhibit activity.

It is important to note that methanethiol is a normal metabolite [19] which can be detected at low levels in the breath of normal individuals [20] but occurs at elevated levels in individuals with liver damage [16,20], including alcoholics. This elevation appears to result from a decrease in the transsulfuration pathway relative to the transamination pathway of methionine metabolism [16,21]. It has also been reported that individuals with alcoholic liver damage [22] exhibit disulfiram-ethanol reaction for much longer periods following discontinuation of disulfiram treatment than normal individuals. Although no explanation for this has been provided [22] it appears likely that the persistence of disulfiram-ethanol reaction in alcoholics with liver damage may be proportional to the production of methanethiol. In this regard, it is especially interesting that the transaminative pathway of methionine degradation is localized in the mitochondria [23], thus facilitating the toxic effects of methanethiol with respect to mitochondrial aldehyde dehydrogenase.

We have demonstrated that the low-K_m mitochondrial isozyme of human aldehyde dehydrogenase can be efficiently inhibited by a potential physiological metabolite of disulfiram: diethyldithiocarbamic acid methanethiol mixed disulfide. The fact that the approach of disulfiram to the essential sulfhydryl group on the mitochondrial isozyme is sterically hindered explains why disulfiram is not an effective inhibitor of the mitochondrial isozyme in vitro despite its overall inhibitory effects on total low-K_m aldehyde dehydrogenase activity in vivo. Since methanethiol occurs naturally, is elevated in alcoholic liver damage [16] and the pathway of its formation is localized in mitochondria [23], it is postulated that diethyldithiocarbamic acid methanethiol mixed disulfide is the in vivo metabolite of disulfiram which inhibits mitochondrial aldehyde dehydrogenase.

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