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# Inhibition of Membrane-Bound Succinate Dehydrogenase By Disulfiram

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## Abstract

The effect of disulfiram on succinate oxidase and succinate dehydrogenase activities of beef heart submitochondrial particles was studied. Results show that disulfiram inhibits both functions. Succinate and malonate suppress the inhibitory action of disulfiram when succinate dehydrogenase is stabilized in an active conformation. Disulfiram is not able to inhibit the enzyme when succinate dehydrogenase is inactivated by oxaloacetate. The inhibitory effect of disulfiram is reverted by the addition of dithiothreitol. From these results, it is proposed that disulfiram inhibits the utilization of succinate by a direct modification of an –SH group located in the catalytically active site of succinate dehydrogenase.

**Key Words:** Disulfiram; antabuse; heart mitochondria; submitochondrial particles; succinate dehydrogenase; sulfhydryl groups.

## Introduction

Disulfiram is an orally active compound that has long been used in the treatment of alcoholism. Since the introduction of disulfiram (Dis) in the management of the alcoholic illness, many *in vivo* and *in vitro* studies have been carried out with the intention of clarifying the mechanism of the disulfiram-ethanol reaction. In this regard, evidence has been provided which indicates that the drug interferes with many important enzyme systems such as liver aldehyde dehydrogenase (Graham, 1951), xanthine oxidase (Richert *et al.*, 1950), and dopamine-$\beta$-hydroxylase (Goldstein *et al.*, 1964). Disulfiram also inhibits $NAD^+$-dependent mitochondrial oxygen consumption and oxidative phosphorylation in rat liver mitochondria (Hassinen, 1966). It has been proposed that the inhibitory action of Dis is related to its ability

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to react with the sulfhydryl groups of proteins to form mixed disulfides (Eneanya *et al.*, 1981; Kelner and Alexander, 1986).

Recently, studies in our laboratory have shown that Dis interferes directly with the elements that establish the mitochondrial calcium balance (Châvez *et al.*, 1989). In order to further characterize the effect of disulfiram on the mitochondrial homeostasis, we studied the influence of the drug on the activities of succinate oxidase and succinate dehydrogenase. The results are discussed in terms of the participation of an –SH group, located in the active site of SDH, in the binding of the inhibitor disulfiram.

## Materials and Methods

Mitochondria from bovine heart were prepared by the polytron technique as reported before (Jurkovitz *et al.*, 1974). Bovine-heart mitochondria were used for the preparation of submitochondrial particles ($ETP_H$) as indicated by Lee and Ernster (1966). As obtained, membrane-bound SDH contained tightly bound oxaloacetate which inactivates the enzyme (Wojtczak, *et al.*, 1969). In order to remove oxaloacetate from the enzyme, the precipitated particles were suspended in a solution containing 200 mM sucrose, 5 mM malonate-Tris (pH 7.3), and 25 mM Hepes, pH 7.3 (5 mg protein per ml). The mixture was incubated for 1 h at 30°C, cooled on ice, and centrifuged. Then, in order to achieve complete removal of malonate, which is a powerful competitive inhibitor of the enzyme, the $ETP_H$ were washed twice in 200 mM sucrose, 25 mM HEPES (pH 7.3) and 500 mM NaBr. Finally the $ETP_H$ were washed with the same buffer without NaBr, since the anion ($Br^-$) itself is inhibitory at high concentrations (Ackrell *et al.*, 1978). The level of activity reached by the malonate-activated $ETP_H$ was not modified by a subsequent incubation with succinate, indicating that the enzyme was fully activated without any bound oxaloacetate (Kearney, 1957; Kearney *et al.*, 1974). Also, the catalytic activity (phenazine methosulfate) measured at 3°C did not show a lag period, indicating that the preparation did not contain malonate (Coles and Singer, 1977).

Analysis of the respiratory rate was carried out polarographically with a Clark-type electrode in a medium containing 200 mM sucrose/25 mM Tris-HCl (pH 7.35). Succinate dehydrogenase activity was measured spectrophotometrically (Mowery *et al.*, 1977) at 25°C in a mixture comprising 200 mM sucrose, 25 mM Tris-HCl (pH 7.35), 5 mM succinate (or the amount needed to complete this concentration in the case of medium already having succinate), 1 mM NaCN, 50 $\mu$M dichloroindophenol (DCIP), and 1.4 mM phenazine methosulfate (PMS). The reaction was started by the addition of an appropriate amount (41.7 $\mu$g/ml) of the $ETP_H$

preparation. Protein was determined according to the method of Lowry *et al.* (1951).

## Results

As demonstrated by the experiment in Fig. 1a, the incubation of $ETP_H$ with 60 $\mu$M disulfiram in sucrose medium at pH 7.35 resulted in a time-dependent loss of succinate oxidase activity. This inhibition could be the consequence of the binding of disulfiram at multiple sites of the respiratory chain. In order to evaluate a direct interaction between disulfiram and SDH, the effect of the incubation of $ETP_H$ with disulfiram on the SDH activity was measured. As observed in Fig. 2, increasing concentrations of disulfiram (10–100 $\mu$M) added to the $ETP_H$ incubation mixture induce a progressive increment on the rate of inactivation of SDH activity.

The inactivation of membrane-bound SDH by disulfiram does not directly imply that a specific residue is being modified nor that such residue

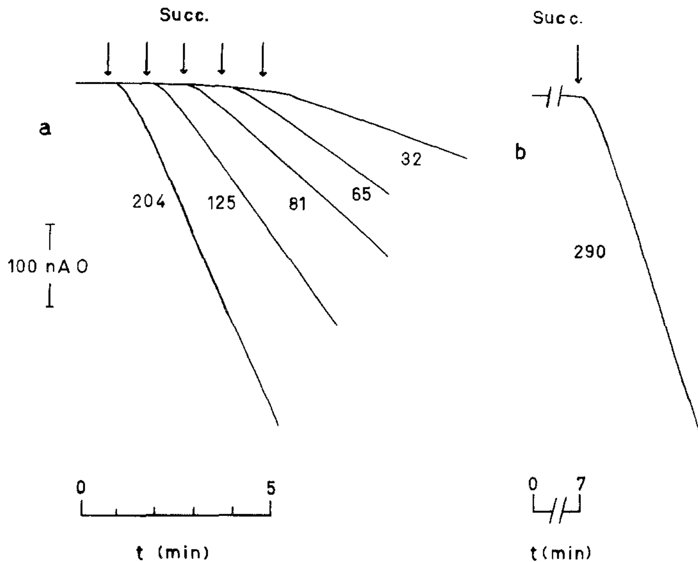


**Fig. 1.** Effect of disulfiram on the aerobic oxidation of succinate. (a) Protein (0.5 mg) from $ETP_H$ was preincubated for different times in a medium (final volume 2 ml) as described in Materials and Methods, which also contained 60 $\mu$M disulfiram. After the preincubation, 5 mM succinate-Tris, pH 7.35 (↓), was added to the mixture in order to determine the remaining rate of oxygen consumption. The activity without added disulfiram was constant during the experiment. (b) The respiratory rate of the control, after preincubation for 7 min in the same medium without added disulfiram, is shown. The numbers indicate the number of atoms of oxygen consumed per minute per milligram of protein. Temperature 25°C.

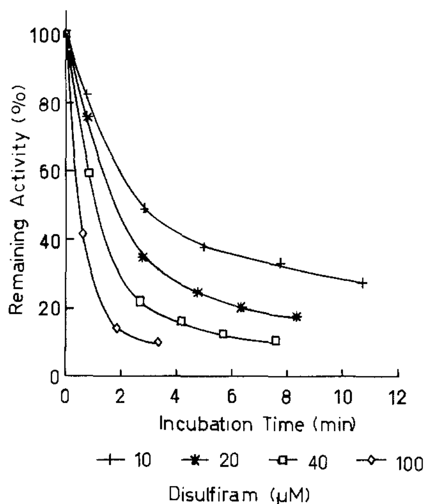


**Fig. 2.** Inactivation of membrane-bound SDH by disulfiram. Submitochondrial particles (43.1 μg/ml) were incubated in a mixture containing 200 mM sucrose, 25 mM Tris-HCl (pH 7.35), 1 mM NaCN, and the indicated concentrations of disulfiram. At the times indicated on the abscissa, the appropriate amount of the $ETP_H$ was withdrawn and their succinate dehydrogenase activity was assayed as described in Materials and Methods. The activity without added disulfiram was constant during the experiment (0.75 μM succinate oxidized per min per mg protein).

lies at the catalytically active site. However, protection of an enzyme by substrate or by competitive inhibitor would suggest that the amino acid residues that are protected belong to the active site. Figure 3 shows that increasing concentrations of succinate (50–400 μM) progressively diminish the rate of enzyme inactivation by disulfiram (50 μM). Similar results with malonate (2.5–20 μM) are shown in Fig. 4. Thus, succinate and malonate protect membrane-bound SDH against inhibition by disulfiram.

The experiments described so far were carried out with $ETP_H$ in which SDH had been previously activated with malonate (see Materials and Methods). However, in mitochondria, succinate dehydrogenase is subjected to a complicated regulatory mechanism (Singer *et al.*, 1973). The enzyme can exist under two forms: an inactive state stabilized by the binding of oxaloacetate and other negative modulators (Wojtczak *et al.*, 1969; Chávez *et al.*, 1986) and an active state stable in the presence of different positive modulators such as succinate or malonate (Kearney, 1957; Kearney *et al.*, 1974; Gutman, 1977).

The question arises whether the inactive form of SDH is also able to readily react with disulfiram to inhibit the enzyme activity. Figure 5 shows that when $ETP_H$ were incubated for 20 min in the presence of oxaloacetate

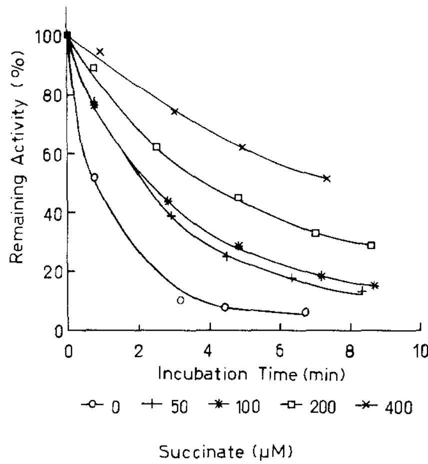


**Fig. 3.** Effect of succinate on the inactivation of membrane-bound SDH by disulfiram. $ETP_H$ were modified by disulfiram (50 $\mu$M) as described in Fig. 2 but in the presence of the indicated concentrations of succinate. In the absence of disulfiram, the enzyme was perfectly stable in the presence or absence of succinate.

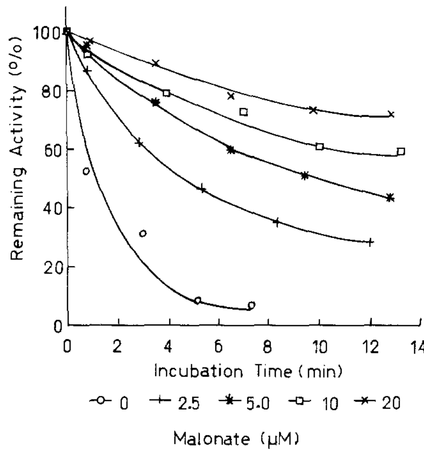


**Fig. 4.** Effect of malonate on the inactivation of membrane-bound SDH by disulfiram. $ETP_H$ were modified by disulfiram (50 $\mu$M) as described in Fig. 2 but in the presence of the indicated concentrations of malonate. In the absence of disulfiram, the enzyme was perfectly stable in the presence or absence of malonate.

and then spun down, the specific activity of the enzyme was very low, indicating that SDH was stabilized under the inactive form (Fig. 5, solid bar, Ox). Under these conditions, reactivation of the enzyme could be achieved by further incubation with succinate (Fig. 5, hatched bar, Ox). When disulfiram was added to oxaloacetate-treated $ETP_H$ and the suspension was centrifuged,

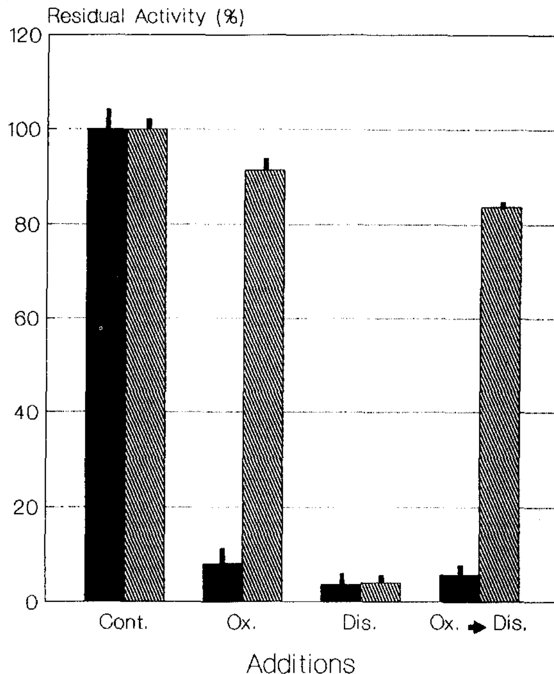


**Fig. 5.** Comparative inhibition of membrane-bound succinate dehydrogenase by disulfiram measured with activated or deactivated enzyme. Membrane-bound SDH (0.5 mg/ml) was activated with malonate as indicated in Materials and Methods or deactivated by incubation for 20 min with 1.0 mM oxaloacetate (Ox). Then, where indicated, 60 $\mu$M disulfiram (Dis) was added and the suspensions were allowed to react for 10 min at 25°C. All the samples were cooled on ice, centrifuged, and their activities measured without (solid bars) or with further activation (hatched bars) for 20 min at 25°C in a medium containing 200 mM sucrose, 25 mM Tris-HCl (pH 7.35), 20 mM succinate, and 1 mM NaCN. Values are mean $\pm$ standard deviation of three experiments.

the recovered activity was also low (Fig. 5, solid bar, Ox → Dis). However, when succinate was added to activate this last preparation, it was observed that the catalytic activity of the reactivated SDH was not inhibited by disulfiram (Fig. 5, hatched bar, Ox → Dis), indicating that the group which is modified by Dis in the active enzyme (Fig. 5, solid bar, Dis and hatched bar, Dis), became uncreactive in the oxaloacetate-inactivated SDH.

Since disulfiram binds to protein sulfhydryl groups (Eneanya *et al.*, 1981; Kelner and Alexander, 1986), the ability of the reducing agent dithiothreithol (DTT) to revert the inhibitory action of Dis was tested. Figure 6 (hatched bar, Dis → DTT) shows that the addition of 1 mM DTT restored 76% of the activity in the $ETP_H$ that had been previously treated with disulfiram.

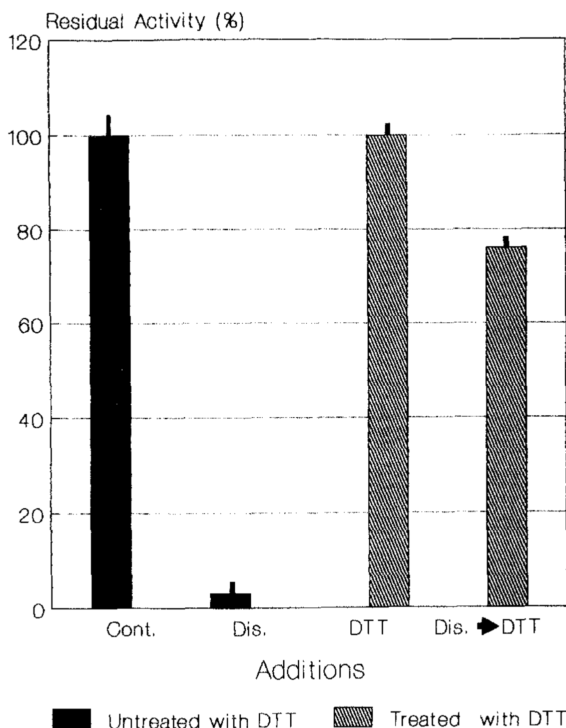


**Fig. 6.** Reversal by dithiothreitol of disulfiram inhibition of succinate dehydrogenase activity. $ETP_H$ (0.5 mg/ml) were incubated in 200 mM sucrose and 25 mM Tris-HCl (pH 7.35). Where indicated, membrane-bound SDH was allowed to react for 10 min at 25°C with 60 $\mu$M disulfiram (Dis). Then, where indicated, 1 mM dithiothreitol was added (DTT) and the reaction allowed to proceed for 4 min more. All the samples were cooled on ice, centrifuged, and their activities measured. Values are mean $\pm$ standard deviation of four experiments.

## Discussion

The evidence presented in this study shows that disulfiram inhibits the oxidation of succinate by submitochondrial particles. As has been indicated, this inhibitory action of Dis parallels the inhibition of the succinate dehydrogenase activity. In this regard, it was found in an early study by Hassinen (1966) on the effect of disulfiram on mitochondrial oxidations that Dis did not affect the rate of oxygen consumption by submitochondrial particles while utilizing succinate as sustrate. Our present results provide an explanation for this apparent discrepancy since, as indicated in Figs. 3 and 4, the presence of ligands of the active site, such as succinate or malonate, in the incubation medium protects the SDH enzyme against the inhibitory action of disulfiram.

The protective effect of succinate and malonate cannot be ascribed to an activation of the enzyme since the above experiments were carried out with fully activated membrane-bound SDH. The results rather suggest that the inhibition of the activated SDH by Dis is the consequence of the modification of a group located in the catalytically active site.

Concerning the nature of the group modified by Dis, it has been reported that disulfiram can react with the protein –SH groups yielding mixed disulfides (Eneanya *et al.*, 1981; Kelner and Alexander, 1986). The finding that dithiothreitol reverts the inhibition induced by Dis strongly suggests that the binding site of the drug is a sulfhydryl group. For succinate dehydrogenase, there is well-documented proof for the presence, in the active site, of an –SH group essential for activity which becomes reactive when the enzyme is stabilized in the active conformation (Kenney, 1975; Vinogradov *et al.*, 1976; Kotlyar and Vinogradov, 1984). Ligands of the active site of SDH prevent the reaction of the –SH group with sulfhydryl-binding reagents, so that it is possible that the active-site –SH group is modified when disulfiram inhibits SDH activity. Supporting this proposal is the fact that the essential –SH group of SDH is not reactive when the enzyme is inactivated by oxaloacetate (Lê-Quôc *et al.*, 1981; Gutman, 1978; Kenney, 1975; Vinogradov *et al.*, 1976; Kotlyar and Vinogradov, 1984). In this regard, it has been suggested that oxaloacetate reacts with the active-site cysteinyl residue via a thiohemiacetal formation (Vinogradov *et al.*, 1971, 1972).

The present results produce better understanding as to the action of disulfiram on the respiratory chain. In addition, they prove the usefulness of Dis as a tool for the study of the active site of SDH. On the other hand, it has to be considered that the inhibition of the Krebs cycle by disulfiram, at the level of SDH, may be one of the important factors contributing to the manifestation of the disulfiram–ethanol reaction. At this point, one may well inquire whether disulfiram can inhibit SDH under all metabolic situations. The data presented here indicate that Dis inhibits the utilization of succinate only when SDH is present in an active conformation. In mitochondria a number of normal constituents (succinate, reduced $CoQ_{10}$, ATP) act as positive modulators of succinate dehydrogenase (Singer *et al.*, 1973). It is at once evident that just when the physiological conditions of the cell permit accumulation of these mitochondrial metabolites, SDH would be vulnerable to disulfiram. In this regard, it is noteworthy that full activation of the enzyme is achieved under conditions of reversed electron flow (Gutman *et al.*, 1971) in which succinate dehydrogenase plays a basic role. This process has been found to be implicated in fatty acid chain elongation (Whereat *et al.*, 1967), a condition otherwise commonly present in the liver during the early stages of alcoholic intoxication (Feinman and Lieber, 1974).

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