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## **DISTINCTION BETWEEN NAD- AND NADH-BINDING FORMS OF MITOCHONDRIAL MALATE DEHYDROGENASE AS SHOWN BY INHIBITION WITH THENOYLTRIFLUOROACETONE**

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### **Summary**

The inhibition of mitochondrial malate dehydrogenase (L-malate : NADH oxidoreductase, EC 1.1.1.37) by 2-thenoyltrifluoroacetone (TTFA) was investigated at pH 8.0 where both forward and backward reactions can be measured.

The inhibition with respect to malate is non-competitive at finite NAD concentrations. Increasing the NAD concentrations lowers the slope of the double reciprocal plot so that at infinite NAD the inhibition is uncompetitive.

The inhibition with respect to oxaloacetate is non-competitive. Increasing the NADH concentration lowers the slope and intercept of the double reciprocal plot so that at infinite NADH the inhibition is nil.

The inhibition with respect to NADH is competitive, whatever the oxaloacetate concentrations are. The inhibition with respect to NAD, at all malate concentrations, is non-competitive.

This pattern of inhibition is incompatible with any model assuming that NAD and NADH reacts with identical forms of the enzyme. On the other hand the reciprocating compulsory ordered mechanism, where the two subunits of the dimeric enzyme are working in concert, can account for all the experimental results.

It is concluded that NAD and NADH bind to different forms of the enzyme separated by reversible steps. Only one form  $\geq E \xrightleftharpoons{\text{NAD}} \text{malate}$ , the one which binds NADH, can react to form the dead end complex  $\text{TTFA} - E \xrightleftharpoons{\text{NAD}} \text{malate}$ .

The similarity between mechanism of inhibition by thenoyltrifluoroacetone and other hydrophobic inhibitors of malate dehydrogenase is discussed.

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### **Introduction**

Previous studies from our laboratory [1] indicated that thenoyltrifluoroacetone (TTFA), once regarded as specific inhibitor of mitochondrial succinate

dehydrogenase [2,3], is an inhibitor of mitochondrial malate dehydrogenase (L-malate : NAD<sup>+</sup> oxidoreductase, EC 1.1.1.37). The inhibition was noted to be uncompetitive with respect to malate and non-competitive with NAD [1] observations which are not suitable for straightforward interpretation considering the complex catalytic mechanism of the enzyme [4–7]. In order to elucidate the mechanism of inhibition the previous studies were extended so as to characterize the nature of the inhibition with respect to all substrates. It is concluded that the inhibition is due to a dead end complex formed between thenoyltrifluoroacetone and the NADH binding form of the enzymes. The inhibition with respect to the other substrates can be explained by Cleland's method [8–10] and the reciprocating compulsory order mechanism of the enzyme [6]. During these studies it becomes apparent that other hydrophobic inhibitors like 1,10-phenanthroline, 1,7-phenanthroline [11] and chlorotricine [12] might inhibit in a similar mode. Thus, the inhibition of malate dehydrogenase by thenoyltrifluoroacetone might be taken as a model for inhibition of this enzyme by a class of hydrophobic inhibitors interacting with the NADH binding form of the enzyme.

## Materials and Methods

**Enzyme.** Mitochondrial pig heart malate dehydrogenase (EC 1.1.1.37) 412 enzyme units/mg (pH 7.5, 25°C) was a Worthington preparation.

**Chemicals.** 2-Thenoyltrifluoroacetone, L-malate and oxaloacetate were obtained for Fluka AG at highest degree of purity. NAD, NADH and Tris were purchased from Sigma.

**Enzymic assay.** Just before use the enzyme was diluted 1 : 1000 in 50 mM Tris/acetate buffer, pH 8.0 (30°C) containing 5  $\mu$ M NADH or NAD in order to stabilize it. The diluted enzyme was kept at 0°C, and a fresh dilution was made every 2 h. The enzyme concentration was determined at 280 nm  $E_{1\text{cm}}^{1\%} = 3.050$  A [16], aliquots of 2.5–40  $\mu$ l were used for assay. Results obtained from different dilution were normalized by running an assay under standard conditions (100  $\mu$ M NADH, 100  $\mu$ M oxaloacetate). Variance between dilutions did not exceed 5%.

**NADH oxidation.** This was measured spectrophotometrically at 340 nm, using Gilford 240. The reaction was carried in 3 ml volume of 50 mM Tris/acetate, pH 8.0, at 30°C. The reaction was initiated by addition of 31 ng of enzyme.

**NAD reduction.** This was measured using a Hitachi MPF2 spectrofluorimeter in the volume and buffer described above at 30°C (excitation 340 nm, emission 460 nm). The reaction was initiated by addition of 496 ng enzyme. Due to quenching of NADH fluorescence by thenoyltrifluoroacetone calibration curves for NADH were run daily at all inhibitor concentrations used the same day.

**Kinetic analysis.** This was carried on a double reciprocal plot. When necessary, least square analysis was carried to verify the positioning of the line.

## Results

### *The effect of thenoyltrifluoroacetone on rate of oxidation of NADH*

The rate of NADH oxidation was measured over a wide concn. range of NADH

(20–100  $\mu\text{M}$ ) and oxaloacetate (30–120  $\mu\text{M}$ ) in the absence and in the presence of thenoyltrifluoroacetone. It should be noted that though the inhibition is quite low, higher concentrations of inhibitor could not be employed due to the strong absorbance of thenoyltrifluoroacetone and 340 nm ( $\lambda_{\text{max}} = 335$  nm,  $\epsilon_{340} = 15.6 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ). The initial velocities were related to the substrate concentration as double reciprocal plots. The intercepts and slopes of this primary plots were used for obtaining the secondary plots given in Fig. 1. Fig. 1A is a plot of the intercepts of the primary plots with respect to  $1/\text{NADH}$ . The inhibition is competitive in nature. This is documented also in Fig. 2 which is Dixon plot for inhibition by thenoyltrifluoroacetone at constant oxaloacetate (197  $\mu\text{M}$ ) and variable NADH (25– $\infty$   $\mu\text{M}$ ). It yields a set of straight lines typical for competitive linear inhibition (Fig. 2).

The inhibition with respect to axaloacetate measured at infinite NADH concentration is practically nil (Fig. 1B). This is in accord with competitive inhibition with respect to NADH. On the other hand, at limited NADH concentrations, the inhibition is appreciable and is non-competitive in nature (Fig. 3). The effect of the inhibitor on the slopes of the primary plots are in accord with these observations. Fig. 4A relates the slopes  $d(v)^{-1}/d[\text{OAA}]^{-1}$  to  $1/[\text{NADH}]$ . At infinite NADH concentration the slopes are identical, but at lower NADH concentrations the inhibitor increases the slope, in accord with the non-competitive inhibition (see Fig. 3). The dependence of  $d(v)^{-1}/d[\text{NADH}]^{-1}$  on  $1/[\text{oxaloacetate}]$  yields two lines (Fig. 4B), compatible with the competitive interaction of thenoyltrifluoroacetone and NADH. The  $K_i$  calculated from the slopes of these figures yields  $K_{i,\text{slope}} = 208 \pm 15$  and  $190 \pm 10 \mu\text{M}$  for Figs. 4A and 4B, respectively.

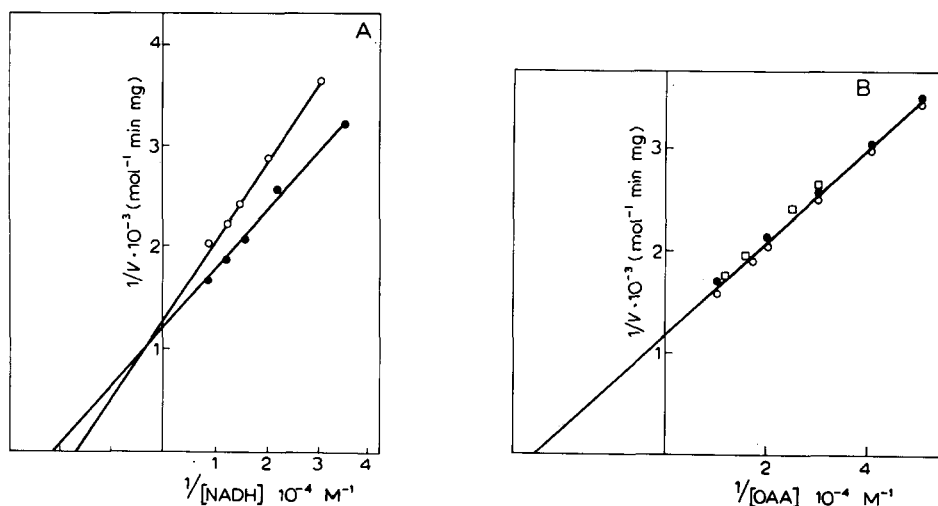


Fig. 1. Secondary plots for the inhibition of NADH oxidation by malate dehydrogenase by thenoyltrifluoroacetone. The results were taken from primary, double reciprocal plots relating the initial velocity with the substrate concentrations. The NADH concentrations ranged from 20 to 100  $\mu\text{M}$  and oxaloacetate varied from 30 to 110  $\mu\text{M}$ . (A) Inhibition with respect to NADH.  $\circ$ , no inhibitor;  $\bullet$ , 96  $\mu\text{M}$  thenoyltrifluoroacetone. (B) Inhibition with respect to oxaloacetate.  $\circ$ , no inhibitor;  $\square$ , 70  $\mu\text{M}$  thenoyltrifluoroacetone;  $\bullet$ , 96  $\mu\text{M}$  thenoyltrifluoroacetone.

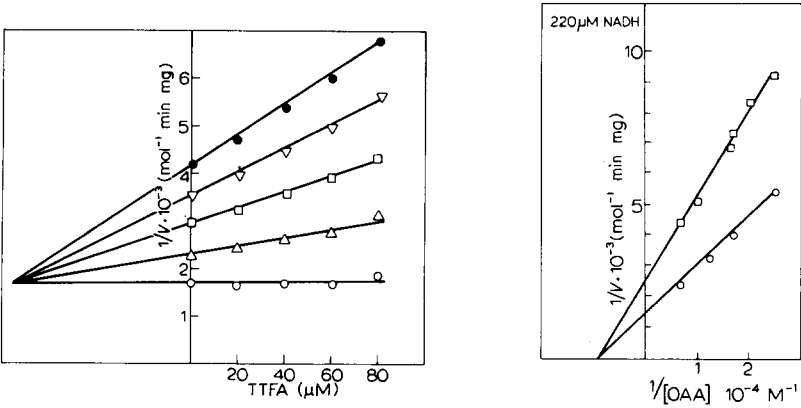


Fig. 2. Dixon plot for inhibition of NADH oxidation by malate dehydrogenase by thenoyltrifluoroacetone at constant oxaloacetate concentrations (197  $\mu\text{M}$ ). NADH:  $\bullet$ , 25  $\mu\text{M}$ ;  $\Delta$ , 33  $\mu\text{M}$ ;  $\square$ , 50  $\mu\text{M}$ ;  $\triangle$ , 100  $\mu\text{M}$ ;  $\circ$ ,  $\infty$ .

Fig. 3. Inhibition of malate dehydrogenase by thenoyltrifluoroacetone at constant NADH concentration (220  $\mu\text{M}$ ).  $\circ$ , no inhibitor;  $\square$ , 111  $\mu\text{M}$ ;  $\circ$ , 160  $\mu\text{M}$ .  $K_i = 160 \mu\text{M}$ .

*The effect of thenoyltrifluoroacetone on rate of reduction of NAD*

Primary double reciprocal plots were measured for the reduction of NAD by malate, in absence and in the presence of inhibitor (not shown). The secondary plots for the inhibition with respect to NAD are given in Fig. 5A. The inhibition is non-competitive with  $K_i = 23\text{--}35 \mu\text{M}$ . The effect of the inhibitor on the slope  $d(v)^{-1}/d[\text{NAD}]^{-1}$  is given in Fig. 5B. At all malate concentrations the slope in presence of the inhibitor is higher than in its absence. This is consistent with non-competitive inhibition with respect to NAD.

The inhibition with respect to malate at infinite NAD concentrations in un-

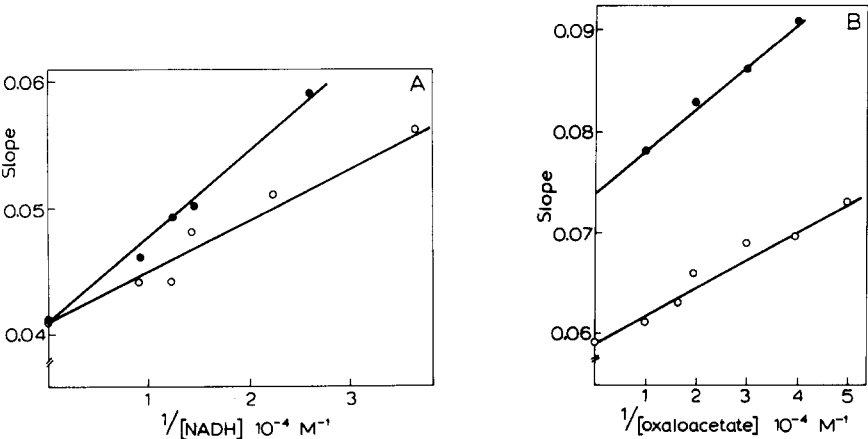


Fig. 4. The effect of thenoyltrifluoroacetone on the slopes of the primary plots for NADH oxidation by malate dehydrogenase. (A) Ordinate  $d(v)^{-1}/d[\text{oxaloacetate}]^{-1}$ ; abscissa  $[\text{NADH}]^{-1}$ . (B) Ordinate  $d(v)^{-1}/d[\text{NADH}]^{-1}$ ; abscissa  $[\text{oxaloacetate}]^{-1}$ .  $\circ$ , no thenoyltrifluoroacetone;  $\bullet$ , 96  $\mu\text{M}$  thenoyltrifluoroacetone. Note discontinuity of the ordinates.

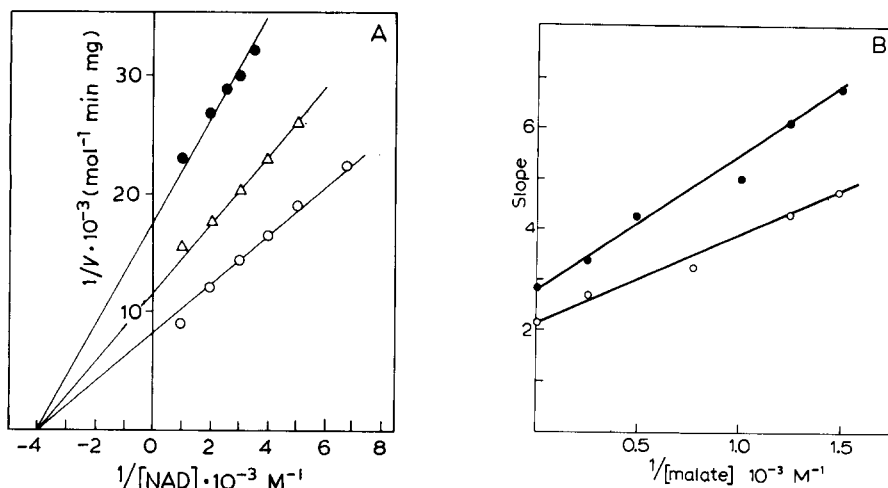


Fig. 5. Secondary plots for the inhibition of by thenoyltrifluoroacetone of NAD reduction by malate dehydrogenase. (A) The effect of thenoyltrifluoroacetone on intercepts of primary plots.  $\circ$ , no inhibitor;  $\Delta$ , 15  $\mu\text{M}$  thenoyltrifluoroacetone;  $\bullet$ , 30  $\mu\text{M}$  thenoyltrifluoroacetone;  $K_i = 20\text{--}25 \mu\text{M}$ . (B) The effects of thenoyltrifluoroacetone on the slope of primary plots. Ordinate  $d(v)^{-1}/d[\text{NAD}]^{-1}$ ; abscissa  $[\text{malate}]^{-1}$ .  $\circ$ , no inhibitor;  $\bullet$ , 15  $\mu\text{M}$  thenoyltrifluoroacetone.

competitive (Fig. 6A) with  $K_i = 11 \mu\text{M}$ . The slopes of primary plots  $d(v)^{-1}/d[\text{malate}]^{-1}$  vs.  $1/[\text{NAD}]$  (Fig. 6B) converge at infinite NAD, but at finite NAD concentrations the slopes are different, suggesting a non-competitive inhibition with respect to malate. Fig. 7 documents that at lower NAD levels this is the case. Finally the Dixon plot for inhibition measured with 4 mM malate and at variable NAD concentrations (Fig. 8) yields a set of straight lines intersecting on the X-axis at 35  $\mu\text{M}$  thenoyltrifluoroacetone.

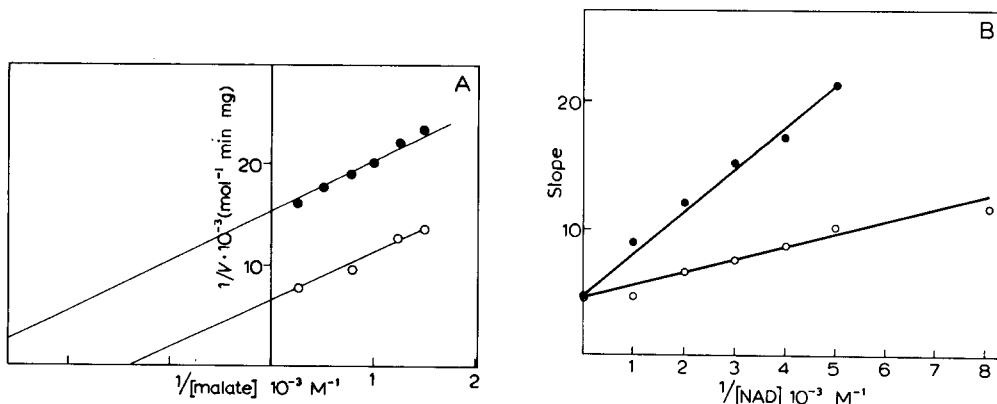


Fig. 6. Secondary plots for the effect of thenoyltrifluoroacetone with respect to malate concentration. (A) The effect of thenoyltrifluoroacetone on intercept of primary plots.  $\circ$ , no inhibitor;  $\bullet$ , 15  $\mu\text{M}$  thenoyltrifluoroacetone;  $K_i = 11 \mu\text{M}$ . (B) The effect of thenoyltrifluoroacetone on the slopes of primary plots. Ordinate  $d(v)^{-1}/d[\text{malate}]^{-1}$ ; abscissa  $[\text{NAD}]^{-1}$ .  $\circ$ , no inhibitor;  $\bullet$ , 15  $\mu\text{M}$  thenoyltrifluoroacetone;  $K_i = 6.4 \mu\text{M}$ .

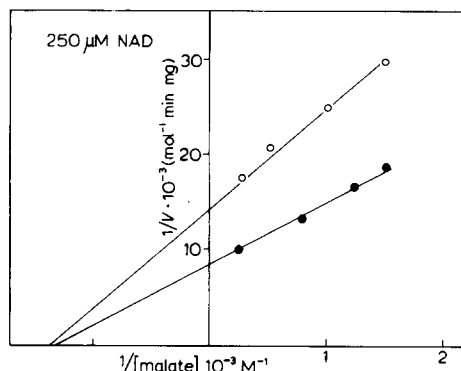


Fig. 7. Inhibition of NADH reduction by thenoyltrifluoroacetone at constant NAD (250 μM). ●, no inhibitor; ○, 15 μM thenoyltrifluoroacetone;  $K_i = 22 \mu\text{M}$ .

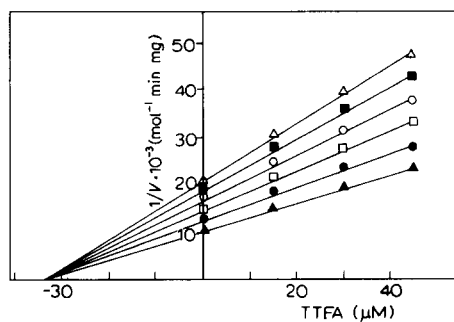


Fig. 8. Dixon plot for inhibition of NAD reduction at constant malate concentration (4 mM) NAD: △, 200 μM; ■, 250 μM; ○, 330 μM; □, 500 μM; ●, 1000 μM; ▲, ∞.

## Discussion

There is a general agreement that malate dehydrogenase (at pH 8) follows an ordered Bi-Bi mechanism where the nucleotides are first to bind and last to leave the enzyme [4–7,13,14], but some peculiarities suggested that the mechanism might be more complex. Heyde and Ainsworth [14] noted a difference between the dissociation constant of NAD and NADH ( $K_{\text{NAD}}$  and  $K_{\text{NADH}}$ ) as derived from kinetic analysis and the  $K_i$  value of these nucleotides as product inhibitors. The conclusion derived was that an isomerisation step of either E-NADH or E-NAD should be considered as a step in the sequence of the reaction. Similar peculiarity with respect to oxaloacetate was also noted [6] where the  $K_{\text{OAA}}$  and  $K_{i,\text{OAA}}$  differed by a factor of 10.

An explanation for these discrepancies was given by introducing the reciprocating compulsory ordered mechanism [6], a model which obeys the same rate equations of an ordered Bi-Bi reaction. According to this model, the two subunits of the enzyme are working in concert, the substrates are added, in an ordered reaction, to one subunit, while the products are released, in ordered sequence, from the second subunit. The catalytic cycle is completed, via unidentified intermediate EXY, (see Scheme 1) by a redox reaction associated with translocation of the substrates from one subunit to the other. Under normal conditions only ternary or higher complexes exist. According to this model the two subunits are interacting with each other by conformational changes so that the substrates bound on one subunit can affect the dissociation constants of the substrates on the other subunit [6]. As a result, though the two subunits are of identical properties and completely symmetric, the form which binds NAD and that which binds NADH are not the same. This dissimilarity is imposed by the substrates bound to the other subunit.

Recent measurements of the effect of adenine nucleotides on fluorescence of NADH bound to malate dehydrogenase indicated that the binding of NADH to the enzyme is at two types of binding sites. Only from one of them was NADH displaced by ADP [15], in accord with the model of Harada and Wolfe

[6]. On the other hand, the reciprocating compulsory ordered mechanism failed to account for exchange reactions measured under equilibrium, neither an evidence for an abortive complex of the nature  $\text{OAA} \xrightarrow{\text{NADH}} \text{E} \xleftarrow{\text{OAA}} \text{NAD}$  was obtained [5]. On this ground, Silverstein and Sulebele [5] rejected the interaction between the two subunits, but the consequences of it is that NAD and NADH must react with the same form of the enzyme. As will be shown in this discussion, the inhibition by thenoyltrifluoroacetone is incompatible with the model of Silverstein and Sulebele [5] and demonstrates that the binding forms for the two nucleotides are not the same.

The Dixon plots for inhibition (Figs. 2 and 8), both for forward and backward reactions are linear, thus we can assume that the inhibitor reacts only with one form of the enzyme [10]. Table I summarizes the modes of inhibition with respect to the different substrates and the type of inhibition predicted according to Cleland [10] for either ordered Bi-Bi [4,5] or reciprocating compulsory ordered mechanisms [6].

As seen from the table, the inhibition of NADH oxidation is compatible with the two mechanisms. Thenoyltrifluoroacetone behaves as competitive inhibitor with respect to NADH, either at infinite (Fig. 1A) or at any other oxaloacetate concentration. This is demonstrated in Fig. 4B, where the inhibitor increases the slope of the reciprocal plots  $d(v)^{-1}/d[\text{NADH}]^{-1}$  at all oxaloacetate concentrations. As competitive inhibition is observed only with respect to NADH it is concluded that the inhibitor and NADH reacts with the same form of the enzyme and E-TTFA is a dead end complex.

The inhibition with respect to oxaloacetate is non-competitive (Fig. 3) indicating that thenoyltrifluoroacetone reacts with another form of the enzyme, separated from oxaloacetate binding form by reversible steps. At infinite NADH concentration inhibition with respect to oxaloacetate obviously disappears due to the competitive nature of the inhibitory complex in this pathway except the one with which NADH competes, in accord with the linearity of the Dixon plot (Fig. 2).

TABLE I

TYPES OF INHIBITION OF MITOCHONDRIAL MALATE DEHYDROGENASE BY THENOYLTRIFLUOROACETONE

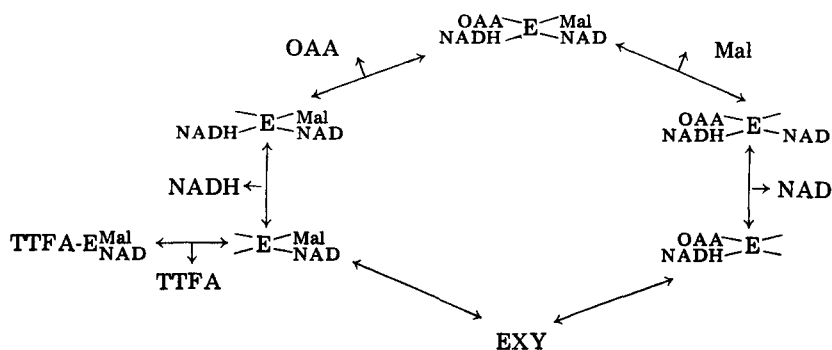
Mode of inhibition predicted according to Cleland [8–10] for ordered Bi-Bi reaction [4,5] assuming E-TTFA to be dead end complex. The mode of inhibition predicted according to Cleland [8–10] for reciprocating compulsory ordered reaction [6]. Assuming TTFA-E<sup>NAD</sup>Mal to be dead end complex C, competitive; NC, non-competitive; UC, uncompetitive, OAA, oxaloacetate.

Variable substrate	Constant substrate	Observed inhibition	Expected inhibition	
			Ordered Bi-Bi mechanism	Receprocating compulsory ordered
NADH	OAA = $\infty$ or finite	C	C	C
Oxaloacetate	NADH = $\infty$	No inhibition	No inhibition	No inhibition
Oxaloacetate	NADH = finite	NC	NC	NC
NAD	Malate = $\infty$ or finite	NC	C	NC
Malate	NAD = $\infty$	UC	No inhibition	UC
Malate	NAD = finite	NC	NC	NC

The inhibition by thenoyltrifluoroacetone of NAD reduction is non-competitive (Figs. 5A and 5B), meaning that the inhibitor and NAD react with different forms of the enzyme separated by reversible steps. As the nature of inhibition does not vary at infinite malate concentrations then, considering the ordered mechanism [4–7], thenoyltrifluoroacetone must react with an enzyme form preceding that which binds NAD. But as NADH and the inhibitor react with the same form, we must conclude that NAD and NADH do not react with the same form of the enzyme.

This conclusion is inconsistent with any model assuming that NADH and NAD react (in the normal pathway of the reaction) with the same form of the enzyme. Were this the case then the inhibition with respect to NAD should have been competitive too. Our findings thus exclude the simple ordered Bi-Bi mechanism as descriptive for the sequence of catalytic cycle.

SCHEME 1.



The reciprocating compulsory mechanism (Scheme 1) can account for our observations. If NADH and thenoyltrifluoroacetone react with the same enzyme form, then the dead end complex will be  $\text{TTFA} \text{--} \text{E} \text{--} \text{Mal} \text{--} \text{NAD}$ . This will yield a competitive inhibition in NADH oxidation, but for NAD reduction, where  $\text{OAA} \text{--} \text{E} \text{--} \text{Mal} \text{--} \text{NAD}$  must be formed in order to bind NAD, a non-competitive inhibition is expected [10]: thenoyltrifluoroacetone reacts with another form of the enzyme thus affecting intercept, but as these are reversible steps it will also affect the slope. The fact that even at infinite malate and NAD concs. the inhibition is still non-competitive (Fig. 5B) means that neither substrates can insert an irreversible step between thenoyltrifluoroacetone and NAD binding forms. The location indicated on the scheme, and the nature of EXY are in accord with this demand.

The inhibition with respect to malate is also compatible with the suggested location of the dead end complex. At low NAD concentrations, the inhibition is non-competitive just as it is with respect to NAD. But, at infinite NAD concentrations which render its binding irreversible, the inhibitor can affect only the intercept and the inhibition becomes uncompetitive [10] (Fig. 6B).

The concentration of the thenoyltrifluoroacetone (or NAD) binding from  $\text{E} \text{--} \text{Mal} \text{--} \text{NAD}$  is a function of the substrates concentrations and the partial rate constants of the reaction. Presently no information is available for the  $\text{E} \text{--} \text{Mal} \text{--} \text{NAD} \rightarrow \text{EXY} \rightarrow \text{OAA} \text{--} \text{E} \text{--} \text{Mal} \text{--} \text{NAD}$  transition. Once it was suggested [16] that nucleotide dissociation



tion (or regeneration of substrate nucleotide binding form) is the rate-limiting step of the overall reaction, but the reciprocating compulsory mechanism cannot distinguish the product nucleotide dissociation from the EXY isomerisation. Present knowledge is insufficient for writing the distribution equation for  $E \rightleftharpoons^{Mal} E$  and concomitantly to calculate the true  $K_i$ . It is apparent that in NADH oxidation higher inhibitor concentrations are needed to inhibit the reaction. It might reflect the lower steady-state concentrations of  $E \rightleftharpoons^{Mal} E$  in presence of NADH and oxaloacetate than in presence of NAD and malate. Furthermore as NADH oxidation can be measured only in presence of NADH its competition with thenoyltrifluoroacetone will always lower the inhibitory effect of thenoyltrifluoroacetone. For these reasons thenoyltrifluoroacetone is a better inhibitor of NAD reduction and much lower concentrations suffice to inhibit this reaction. The  $K_i$  values derived for this reaction, measured at saturating NAD and malate (6–11  $\mu$ M) (Figs. 5A and 5B) might be taken as the best approximation for  $K_i$ .

The last point for discussion concerns other inhibitors of malate dehydrogenase reported to have similar effects. These are 1,10-phenanthroline [11] chlorotricin [12] and *p*-bromophenol [16] all inhibit competitively with respect to NADH. The hydrophobic nature of these compounds, similar to thenoyltrifluoroacetone, and the competition with NADH might suggest that their mode of inhibition is similar to that described above. If this is the case then the inhibition by all these compounds might be due to their interaction with a hydrophobic region of the enzyme, probably the one which binds the adenine moiety of the NADH. This type of interaction will imitate the competitive inhibition with respect to NADH reported for adenine nucleotides [15].

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