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INHIBITION OF MALATE DEHYDROGENASE BY THYROXINE AND STRUCTURALLY RELATED COMPOUNDS *

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Summary

The inhibition of pig heart mitochondrial malate dehydrogenase (L-malate : NAD^+ oxidoreductase, EC 1.1.1.37) by thyroxine and structurally related compounds was studied to resolve a longstanding question about the exact nature of the inhibition. Thyroxine, in freshly prepared solution, was found to be a "pure" competitive inhibitor relative to the nucleotide cofactor. Upon standing in diffuse daylight, solutions of thyroxine showed increased ability to inhibit the enzyme, presumably as a result of oxidation of enzyme sulfhydryl groups by free iodine that is released photochemically. This behavior probably accounts for earlier reports of irreversible inactivation by thyroxine. Comment is made on the implications of these findings to the mechanism of thyroid hormone action.

Introduction

Thyroxine has been shown to inhibit the enzymatic activity of a large number of dehydrogenases [1]. The enzyme most sensitive to this inhibitory effect is mitochondrial malate dehydrogenase (L-malate : NAD^+ oxidoreductase, EC 1.1.1.37) which is inhibited by near physiological concentrations of thyroxine. An understanding of the exact nature of the effects observed in the interaction of the enzyme with thyroxine may therefore be important to our understanding of the mechanism of thyroid hormone action [1—6]. Previous studies suggest that the effect of thyroxine may be equated with the effects of iodine and cyanogen iodide on the enzyme [2—4] but the interpretations are internally inconsistent and conflict with an earlier study [1]. Because of these inconsistencies, the interaction of the enzyme with thyroxine and structurally related compounds was reexamined.

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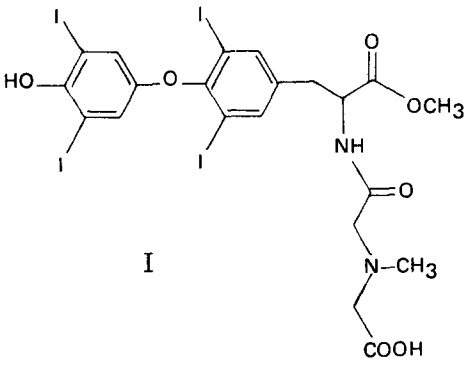
Experimental Procedures

Materials and methods

Thyrosine, 3,3′,5-triiodothyronine, and malic acid were obtained from Sigma. The preparation of methyl thyroxinate and methyl (*N*-methyl-*N*-carboxymethylglycyl)thyroxine (I) will be reported.



I

elsewhere (Ullman et al., unpublished). Mitochondrial malate dehydrogenase from porcine heart was obtained from Miles Laboratories and further purified by gel and ion-exchange chromatography on Sephadex G-100 and DEAE-Sephadex (Pharmacia) by a procedure adopted from that of Glatthaar et al. [7]. Rabbit serum albumin was also purchased from Miles Laboratories.

Kinetic studies

Measurements of initial velocities were carried out using pH 9.4, 0.05 M sodium glycinate buffer containing 0.01% EDTA and 1 μg/ml rabbit albumin. All reactions were carried out in the malate to oxaloacetate direction. Measurements were made with a Bausch and Lomb Spectronic 20 equipped with a Hewlett-Packard X-Y recorder. 1-cm pathlength cuvettes were pre-equilibrated in a water bath kept at 30 ±1°C for at least 5 min before transfer to the spectrophotometer. Protein concentrations were determined from the absorbance at 280 nm using an extinction coefficient of $E^{1\%}_{280\text{nm}} = 2.94$, determined by dry weight of the purified enzyme and a molecular weight of 70 000 [8].

Solutions of thyroxine were prepared in 0.05 M NaOH. Methyl thyroxinate and I were prepared in 0.1 M NaOH containing 5% dimethyl formamide. All solutions were prepared immediately before use and stored at 0° C in the dark during the experiments. 0—5 μl of inhibitor solution were diluted into a total assay volume of 3.2 ml.

Results and Discussion

The inhibition of malate dehydrogenase by thyroxine was first shown to be competitive in nature by Wolff and Wolff [1] who postulated that the inhibition may have in vivo significance in metabolic control. In contrast, Varrone et al. [4] subsequently reported that thyroxine irreversibly inhibits the enzyme by direct oxidation of a thiol group of the enzyme by thyroxine. The extent of

inhibition was reportedly related to the number of thiol groups oxidized, and complete inactivation occurred when all 14 groups were oxidized [4]. The authors compared the inhibitory effect of thyroxine to that of ICN and iodine and found that while both ICN and iodine react stoichiometrically with the thiol groups of the enzyme 7.5 equivalents per sulfhydryl group were required for thyroxine-inactivation of the enzyme. Left unexplained were the unusual stoichiometry of the reaction, the unprecedented ability of thyroxine to behave as an oxidant, and the fate of the reduced thyroxine. Moreover, there were certain inconsistencies in the kinetic data [2—4], the most notable of which was the finding that ICN is simultaneously a competitive inhibitor and an irreversible inhibitor of the enzyme. These considerations, plus the fact that, in the earlier studies of Wolff and Wolff [1], it was found necessary to preincubate the enzyme with thyroxine, raised serious questions about the validity of any of the prior conclusions concerning the inhibition of the enzyme by thyroxine.

Lineweaver-Burk and Dixon plots of the inhibition of malate dehydrogenase by thyroxine are given in Figs. 1 and 2. Table I summarizes the K_i values for thyroxine and related compounds in the presence of different rabbit serum albumin concentrations. Except for triiodothyronine, the inhibition by all the other derivatives was found to be strictly competitive with NAD. Rabbit serum albumin used to stabilize the enzyme in the assay was found to have no effect upon the inhibition constant. The zero intercepts of the Lineweaver-Burk plots and the linear Dixon plots indicate that the tetraiodothyronine derivatives are "pure" competitive inhibitors of the enzyme and no kinetically competent enzyme-substrate-inhibitor complexes are formed [9].

A study was performed to verify directly the reversibility of the thyroxine

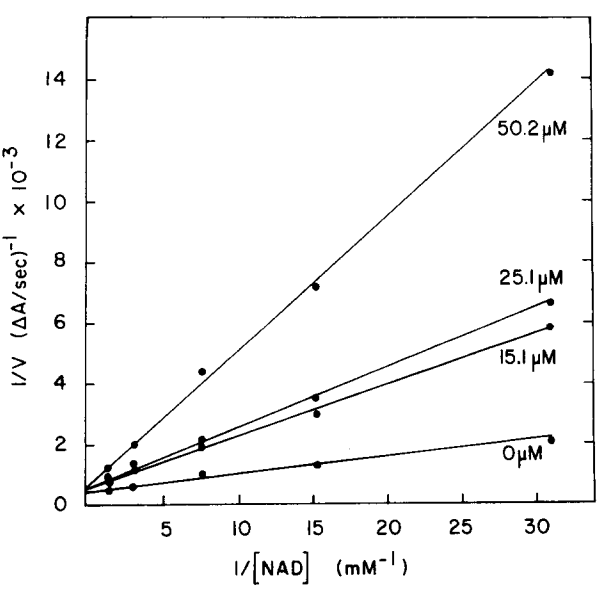


Fig. 1. Inhibition of malate dehydrogenase by different thyroxine concentrations. Initial rates determined with 1.65 nM enzyme in pH 9.4, 0.1 M glycine buffer containing 0.62 mM malate and 1 μg/ml rabbit albumin.

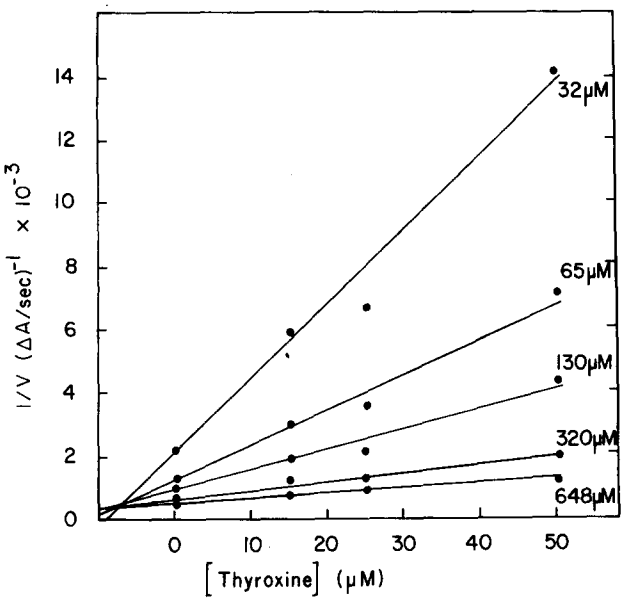


Fig. 2. Dixon plots of inhibition of malate dehydrogenase by thyroxine at different NAD concentrations. Initial rates determined with 1.65 nm enzyme in pH 9.4, 0.1 M glycine buffer containing 0.62 mM malate and 1 μg/ml rabbit albumin.

malate dehydrogenase interaction. A solution the enzyme was incubated in the dark for 2 and 4 h at 4°C with 99.8 μM thyroxine. Control solutions of the enzyme with no thyroxine were incubated simultaneously under identical conditions. A 5-μl aliquot of each solution was then diluted into 3.2 ml of the assay buffer containing either 100 μM thyroxine or no thyroxine. The results, given in Table II, show that the velocity of the enzyme catalyzed reaction was not affected by pre-incubation of the enzyme with thyroxine under various conditions. Inhibition was completely reversible even when rate measurements were made within 30 sec after dilution. When the enzyme was diluted into buffer containing thyroxine at the same concentration as used during the timed incubations, the expected inhibition was observed. When thyroxine solutions were exposed to diffuse daylight the inhibition was dependent on the length of

TABLE I

COMPETITIVE INHIBITION OF MALATE DEHYDROGENASE BY THYROXINE AND RELATED COMPOUNDS IN pH 9.4, 0.1 M GLYCINE BUFFER, 30°C

Inhibitor	[Malate] (mM)	K_i (μM)
Thyroxine	0.62	10
Thyroxine *	0.62	8, 10
Thyroxine **	2.26	9.8
Methylthyroxinate	0.62	10
I	0.62	10
Triiodothyronine ***	0.31	90

* Includes 1 μg/ml rabbit albumin.
** Includes 1 mg/ml rabbit albumin.
*** Partially noncompetitive.

TABLE II

INITIAL VELOCITIES (μM/min) UPON DILUTION OF PREINCUBATED MIXTURES OF THYROXINE AND MALATE DEHYDROGENASE

Preincubation was in dark at 4°C in 0.72 mM malate, pH 9.4, glycine buffer.

Incubation conditions			Rate (μM/min) *	
Time (min)	[Thyroxine] (μM)	[Enzyme] (μM)	0.156 μM Thyroxine in assay	99.8 μM Thyroxine in assay
30	0	0.537	3.1 **	0 **
30	99.8	0.537	3.1 **	0 **
120	0	1.08	5.9 **	1 **
120	99.8	1.08	5.9 **	1 **
240	0	2.15	69 ***	3.1 ***
240	99.8	2.15	69 ***	2.8 ***

* Preincubated solution diluted 640-fold into the above malate/glycine buffer with or without added thyroxine.

** 10 min after dilution the reaction was initiated by adding NAD up to 0.031 mM concentration.

*** Immediately (<30 s) after dilution the reaction was initiated by adding NAD up to 0.31 mM concentration.

exposure (Table III). This was expected since free iodine was formed as demonstrated by its ability to be bleached with thiosulfate [10]. Iodine was shown readily to inactivate the enzyme irreversibly as had been previously reported [2–4].

These findings strongly suggested that the irreversible inhibition by thyroxine reported by Varrone, et al. [2–4] was due to contamination of thyroxine by iodine. Although this inhibitory effect was ascribed to the oxidation of sulfhydryl groups by thyroxine, 7.5 thyroxines were required to oxidize one group. Calculation of the pseudo-first order rate constants for inactivation of the enzyme by iodine, ICN, and thyroxine, using the data presented by Varrone et al. [3] shows that the rate constants for inactivation by iodine and ICN are the same but are 8.3 times the rate constant for irreversible thyroxine inhi-

TABLE III

INHIBITORY EFFECT OF THYROXINE SOLUTIONS ON MALATE DEHYDROGENASE ACTIVITY AFTER EXPOSURE TO INDIRECT DAYLIGHT

Exposure time (h) *	[NAD] (μM)	Rate (μM/min) **	% Increased inhibition
0	630	31.2	
1	630	24.9	20
0	320	24.8	
1	320	20.5	17
5	320	16.2	34

* A 16.1 mM solution of thyroxine in 0.05 M NaOH was exposed to indirect daylight filtered through window glass.

** Rate measurements were made by addition of the thyroxine solution to 0.72 nM malate dehydrogenase and 3.12 mM malate in pH 9.4, 0.1 M glycine buffer so as to give a 99.8 μM thyroxine concentration (assuming no photodecomposition of the inhibitor). The reaction was immediately initiated by addition of NAD.

bition. This is very similar to the 7.5 thyroxine stoichiometry required for sulfhydryl group oxidation which further supports the conclusion that irreversible inactivation of the enzyme was due to the presence of free iodine in the thyroxine sample.

Modification of the α-carboxyl and amino groups of thyroxine has a negligible effect upon the type or strength of inhibition of malate dehydrogenase (Table I). The iodinated phenyl groups thus appear to be responsible for the very tight binding to the enzyme. However triiodothyronine, which has one less iodine atom, is less tightly bound and unlike the tetraiododerivatives may form a catalytically active complex. The enhanced binding produced by the additional iodine is consistent with the observed correlation of electronegativity and hydrophobicity of substituted phenols with their inhibitory effect on the enzyme [11].

Conclusion

The findings of the present study substantiate the earlier finding of Wolff and Wolff [1] that thyroxine is a competitive inhibitor of malate dehydrogenase and illustrate that the preincubation of the enzyme and inhibitor employed by these authors was not required if pure thyroxine is employed. The irreversible inhibition subsequently reported [4] to be the result of oxidation of the thiol groups appears to be an artifact arising from impurities, probably iodine, present in the thyroxine. The mediation of malate dehydrogenase activity by thyroxine proceeds by means of a reversible association with the enzyme. The magnitude of the equilibrium constant for this association together with the normal serum levels of free thyroxine are significant as regards the extent of this mediation in vivo. While the total serum thyroxine level present in mammals is approximately $4 \cdot 10^{-8}$ M [12] the normal free thyroxine concentration is only about $3 \cdot 10^{-11}$ M [13]. Since this is 5 orders of magnitude lower than the K_i of the enzyme for thyroxine significant inhibition of the enzyme by thyroxine is unlikely. Moreover triiodothyronine, which exhibits greater physiological activity [14] is a ten-fold poorer inhibitor although the free hormone is present in a similar concentration to that of free thyroxine [13]. Thus there is little evidence that tri- or tetraiodothyronines can exert their metabolic control by means of a direct interaction with the enzyme as had been previously considered [1—6]. However the possibility cannot be discounted that elevated intracellular thyroxine and/or triiodothyronine concentrations may arise from active transport and result in mediation of the enzyme activity.

Acknowledgement

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