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SUBSTRATE REQUIREMENT FOR INACTIVATION OF IODOTHYRONINE-5'-DEIODINASE ACTIVITY BY THIOURACIL

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Summary

Preincubation of rat liver microsomal fraction with 1 μM 2-thiouracil and 0.01—1 μM 3,3',5'-triiodothyronine or 3',5'-diiodothyronine, 0.1—10 μM thyroxine or 3,5-diiodothyronine led to a progressive, irreversible and concomitant decrease in subsequently assayed 3,3',5'-triiodothyronine- and 3',5'-diiodothyronine-5'-deiodinase activity Preincubation with thiouracil alone, with iodothyronines alone or with thiouracil and 10 μM thyronine or 3,5-diiodotyrosine had no or virtually no effect. The results indicate that (1) a previously proposed ping-pong mechanism for thyroid hormone deiodination, involving the formation of an enzyme-sulphenyl iodide intermediate, is correct, (2) thyroxine, 3,3',5'-triiodothyronine and 3',5'-diiodothyronine are substrates for a common 5'-deiodinase, (3) this 5'-deiodinase is not fully specific as regards the position of the iodine substituents in the substrate, since it also appears to catalyse the 5-deiodination of 3,5-diiodothyronine.

Introduction

The main route of metabolism of iodoamino acids, both iodotyrosines and iodothyronines, is by means of deiodination. In both instances the enzymatic reaction is a reductive process, yet different types of enzymes are involved. Deiodination of iodotyrosines is catalysed by an enzyme complex, where reductive equivalents are supplied by NADPH [1]. Iodothyronine deiodinase activity is also located in the microsomal fraction of several tissues, but present evidence indicates that it constitutes a single enzyme, where reductive equivalents are supplied by thiols [2,3]

In the deiodination of 3,3',5,5'-tetraiodothyronine (thyroxine) two types of reaction may be distinguished, i.e., deiodination of the phenolic ring (5'-

deiodination) yielding 3,3',5-triiodothyronine, and deiodination of the tyrosyl ring (5-deiodination), yielding 3,3',5'-triiodothyronine. Both 3,3',5-triiodothyronine and 3,3',5'-triiodothyronine are subject to further degradation by both 5- and 5'-deiodination. Of all occurring iodothyronines 3,3',5-triiodothyronine is biologically the most active, suggesting the deiodination of thyroxine as a possible site for the regulation of thyroid hormone activity at the level of peripheral tissues. It has been suggested [4] that 5- and 5'-deiodinations are mediated by separate enzymes (iodothyronine 5- and 5'-deiodinase). As yet no direct evidence has been presented to support this hypothesis. On the contrary, subcellular fractionation [5] and subjection of detergent extracts of microsomes to various analytical techniques [3] have failed to separate 5- and 5'-deiodinase activity.

Recent findings [6—9] suggest that deiodination follows a ping-pong mechanism This involves the transfer of an iodinium ion from the substrate to a sulphhydryl group of the enzyme leading to the formation of an enzyme-sulphenyl iodide (E-SI) complex. The E-SI intermediate is subsequently reduced by mercapto compounds (cofactor) to free enzyme. The enzyme is inhibited by derivatives of 2-thiouracil as these compounds react with the E-SI intermediate forming an enzyme-thiouracil mixed disulfide (dead-end complex). This is substantiated by the finding that inhibition by thiouracil is uncompetitive with substrate and competitive with cofactor. Thus, thiouracil only reacts with the enzyme after formation of the E-SI intermediate and, therefore, only if substrate is present. This appears to be true as binding of radioactive thiouracil to rat liver microsomal fraction was specifically induced by substrates of the 5'-deiodinase [7,10]. Moreover, the irreversible inactivation of thyroxine-5'-deiodinase activity by thiouracil was potentiated by thyroxine [7].

Previous experiments have indicated that 5'-deiodination of several iodothyronines is mediated by a single enzyme. Thus, 3,3',5'-triiodothyronine is a competitive inhibitor of the 5'-deiodination of thyroxine and vice versa where values for K_m and K_i were found to be identical [11,12]. Furthermore, the effect of pH on the 5'-deiodination of 3,3',5'-triiodothyronine and 3',5'-diiodothyronine was found to be very similar [13]. Based on the above model this has now been tested more directly by studying the effects of preincubation of enzyme with thiouracil in the presence or absence of several iodothyronines on the subsequent assay of 5'-deiodinase activity. Enzyme activity was destroyed by coincubation with thiouracil and 3,3',5'-triiodothyronine, 3',5'-diiodothyronine, thyroxine or, though to a lesser extent, also with 3,5-diiodothyronine but not with 3,5-diiodotyrosine or thyronine. These results indicate that thyroxine, 3,3',5'-triiodothyronine and 3',5'-diiodothyronine are indeed substrates for a common 5'-deiodinase. The specificity of this enzyme, however, is not complete since it also appears to catalyse, though with lesser efficiency, the deiodination of 3,5-diiodothyronine.

Materials and Methods

Materials

L-Thyronine, 3,5-diiodo-L-thyronine, 3',5'-diido-L-thyronine,3,3',5'-triiodo-

L-thyronine, L-thyroxine and 3,5-diiodo-L-tyrosine were of the highest purity available and were purchased from Henning Berlin GmbH., F.R.G. The purity of 3,5-diiodothyronine was checked by high-performance liquid chromatography. It was found to contain no (<0.1%) 3',5'-diiodothyronine or 3,3',5'-triiodothyronine (T.J. Visser, unpublished data)

Methods

Rat liver microsomal fraction was prepared essentially as described [2]. Aliquots of this preparation (final protein concentration 400 μg/ml) were incubated at 37°C with 1 μM thiouracil and various concentrations of iodoamino acids or thyronine in 0 05 M sodium phosphate/3 mM EDTA/0 1 mM dithiothreitol (pH 6.5). After 30 min the reaction mixtures were diluted 10-fold by the addition of 0.05 M sodium phosphate/3 mM EDTA (pH 6.5) at 0°C. In control experiments, microsomes were incubated without iodoamino acids or thyronine which were added only after the dilution of the reaction mixtures. The resulting suspensions were kept at 0°C until the second incubation. For this, aliquots of the mixtures were incubated at 37°C with equal volumes of 0.05 M sodium phosphate/3 mM EDTA/2 mM dithiothreitol (pH 6.5) containing 2 μM 3',5'-diiodothyronine or 0.5 μM 3,3',5'-triiodothyronine. After 15 min the reaction was stopped by the addition of 9 vol. 0.06 M sodium barbitone/0 15 M NaCl/0.1% bovine serum albumin/0 1% sodium dodecyl sulphate (SDS) (pH 8.6) at room temperature. In controls, substrate was added only after the SDS buffer. The products formed (3'-iodothyronine and 3,3'-diiodothyronine, respectively) were measured in duplicate directly in 50 μl of the extracts by specific radioimmunoassays [12,13].

In a similar type of experiment microsomes were preincubated for 30 min at 37°C with 1 mM *N*-ethylmaleimide and 0.1—1 μM 3',5'-diiodothyronine. The reaction mixtures were now diluted with 0.05 M sodium phosphate/3 mM EDTA/2 mM dithiothreitol (pH 6.5) to block unreacted *N*-ethylmaleimide. The subsequent assay of 5'-deiodinase activity with 3',5'-diiodothyronine as the substrate was performed as described above.

Results

Preincubation of rat liver microsomal fraction with thiouracil alone or with iodothyronines alone did not affect the 5'-deiodination of 3,3',5'-triiodothyronine or 3',5'-diiodothyronine in the second incubation. However, coincubation of microsomes with 1 μM thiouracil and 0.01—1 μM 3,3',5'-triiodothyronine, 0.01—1 μM 3',5'-diiodothyronine, 0.1—10 μM thyroxine or 0.1—10 μM 3,5-diiodothyronine resulted a progressive, irreversible loss of 3,3',5'-triiodothyronine-5'-deiodinase activity (Fig. 1) After incubation of microsomes with thiouracil in the presence of 10 μM thyronine or diiodotyrosine, 3,3',5'-triiodothyronine-5'-deiodinase activity was found to be 91 ± 9% and 80 ± 7% (mean ± S D , n = 6) of the control value, respectively The presence of as low as 0.01 μM 3,3',5'-triiodothyronine or 3',5'-diiodothyronine in the preincubation resulted in a loss of 60% of enzyme activity in both cases The addition of 0 1 μM thyroxine or 1 μM 3,5-diiodothyronine to the reaction mixtures during the preincubation led to irreversible decrease in 5'-deiodinase activity by 40 and

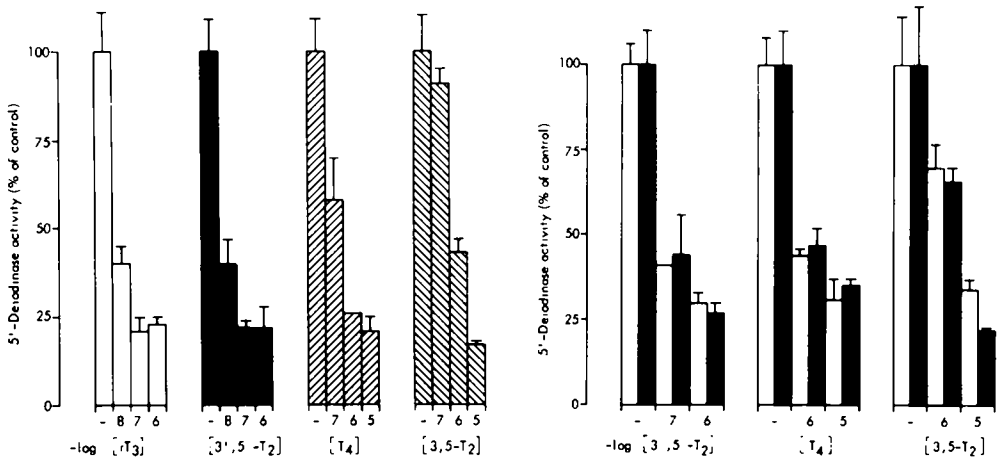

Fig. 1. Effect of preincubation of rat liver microsomal fraction with 1 μM thiouracil with or without various concentrations of 3,3',5'-triiodothyronine (rT$_3$), 3',5'-diiodothyronine (3',5'-T$_2$), thyroxine (T$_4$) or 3,5-diiodothyronine (3,5-T$_2$) on the subsequent assay of 3,3',5'-triiodothyronine-5'-deiodinase activity. For details see text Results are expressed as percentage of control and are given as mean ± S D (n = 4—8)

Fig. 2 Effect of preicubation of rat liver microsomal fraction with 1 μM thiouracil with or without various concentrations of 3',5'-diiodothyronine (3',5'-T$_2$), thyroxine (T$_4$) or 3,5-diiodothyronine (3,5-T$_2$) on the subsequent assay of 3,3',5'-triiodothyronine-5'-deiodinase (□) and 3',5'-diiodothyronine 5'-deiodinase (■) activity For details see text. Results are expressed as percentage of control and are given as mean ± S D (n = 4—8).

60%, respectively (Fig. 1). Under the conditions tested, maximum inhibition was obtained with 0.1 μM 3,3',5'-triiodothyronine or 3',5'-diiodothyronine, 1 μM thyroxine and 10 μM 3,5-diiodothyronine and amounted to approx. 80%. Fig. 2 shows that coincubation of microsomes with 1 μM thiouracil and increasing concentrations of 3',5'-diiodothyronine, thyroxine or 3,5-diiodothyronine led to a progressive, parallel loss of 3',5'-triiodothyronine- and 3',5'-diiodothyronine-5'-deiodinase activity. Irrespective of the substrate used in the second incubation, virtually maximum inhibition was reached again using 0.1 μM 3',5'-diiodothyronine, 1 μM thyroxine or 10 μM 3,5-diiodothyronine, and in these experiments amounted to approx. 70%.

Even after the subsequent addition of excess dithiothreitol, 3',5'-diiodothyronine-5'-deiodinase activity was found to be greatly inhibited following the reaction of microsomes with 1 mM *N*-ethylmaleimide. Compared with the control experiment, where *N*-ethylmaleimide was added only after the dithiothreitol, only 4—5% of deiodinase activity was left. The presence of 0.1 or 1 μM 3',5'-diiodothyronine during the preincubation did not protect against the inhibitory activity of *N*-ethylmaleimide. This resulted only in an increase in the subsequent 5'-deiodination of 3',5'-diiodothyronine to 7—8% of the control.

Discussion

The conditions for the experiments described in the previous section were chosen such that concentrations of thiouracil and iodothyronines were high enough in the first incubation to ensure a proper interaction with the enzyme. After dilution, the concentrations of these compounds would need to be decreased as much as to prevent the interference with the subsequent assay of deiodinase activity. Previous studies had shown that 0.1 and 1 μM thiouracil inhibits enzyme activity by 25 and 75%, respectively, in reaction mixtures containing 0.1 μM 3,3',5'-triiodothyronine (substrate)/1 mM dithiothreitol (cofactor) at pH 6.5 [14]. In the present experiments, the mixtures contained 1 μM thiouracil/0 1 mM dithiothreitol in the first, and 0 05 μM thiouracil/1 mM dithiothreitol in the second incubation. At pH 6.5 in the presence of 3 mM dithiothreitol, K_m values for thyroxine, 3,3',5'-triiodothyronine and 3',5'-diiodothyronine are 3, 0.04 and approx. 0.1 μM, respectively (Refs. 12, 13, D. Fekkes, E. van Overmeeren and T.J. Visser, unpublished work). Deiodination rates of 3,3',5'-triiodothyronine and 3',5'-diiodothyronine are also much higher compared with thyroxine [13] The concentrations of 3,3',5'-triiodothyronine, 3',5'-diiodothyronine and thyroxine were at the most 1, 1 and 10 μM in the first, and 0.05, 0.05 and 0.5 μM, respectively, in the second incubation. In the presence of saturating concentrations of substrate in the second incubation, the little amount of iodothyronine carried over from the first incubation would, therefore, not interfere with estimation of enzyme activity. These considerations were borne out by the findings that enzyme activity measured after incubation of microsomes with thiouracil alone or with iodothyronine alone was not different from the control experiment.

It has been shown previously that in the presence of substrate the reaction of thiouracil with the 5'-deiodinase leads to an irreversible inactivation of the enzyme [6—8]. This is probably the result of the formation of an enzyme-thiouracil mixed disulfide by reaction of thiouracil with an enzyme-sulphenyl iodide (E-SI) intermediate. Leonard and Rosenberg [7] also found some inhibition by propyl-thiouracil (PTU) in the absence of substrate. This discrepancy with our observations may be due to different experimental conditions, as these workers used high concentrations of PTU (10 μM compared with 1 μM thiouracil used in our study) in the pre-incubation, and low concentrations of dithiothreitol (0.1 mM vs. 1 mM used here) to assay 5'-deiodinase activity. We have now demonstrated that preincubation of rat liver microsomal fraction with thiouracil and low concentrations of 3,3',5'-triiodothyronine, 3',5'-diiodothyronine or thyroxine results in a decrease in the subsequently assayed 5'-deiodinase activity. These findings not only confirm the proposed mechanism of inhibition by thiouracil, but also prove that indeed these iodothyronines are substrates for a common 5'-deiodinase. This is substantiated once more by the observation that the 5'-deiodinations of 3,3',5'-triiodothyronine and 3',5'-diiodothyronine were affected to very similar extents depending on the conditions during the preincubation. The specificity of the inactivation process is illustrated by the finding that the inhibitory activity of thiouracil is not or virtually not expressed in the presence of high concentrations of thyronine or diiodotyrosine. Moreover, the relative activity of thyroxine, 3,3',5'-tri-

iodothyronine and 3',5'-diiodothyronine in the induction of dead-end complex formation by thiouracil mirrors their performance as substrates for the 5'-deiodinase. This is further support for the concept that compounds which assist in the inactivation of the 5'-deiodinase by thiouracil are also substrates for this enzyme.

Thus, there appears to be an absolute requirement for the presence of substrate in the irreversible inactivation of the 5'-deiodinase by thiouracil. Unexpectedly, 3,5-diiodothyronine also fulfilled this requirement, despite the absence of iodine substituents in the 3' and 5' positions. From these results it is inevitable to conclude that 3,5-diiodothyronine is also deiodinated by the same enzyme which mediates the 5'-deiodination of other iodothyronines. Both 5- and 5'-deiodinations may, therefore, be catalysed by the same enzyme. Whether this implicates that only a single enzyme is involved in the entire sequential deiodination of thyroxine remains to be investigated. Published observations of the deiodination of thyroid hormone in vivo and in vitro are best explained by the two-enzyme hypothesis. If this hypothesis proves to be correct our results indicate that these enzymes do not display full specificity. Recent studies have also shown that at high concentrations 3,5-diiodothyronine is a competitive inhibitor of the 5'-deiodination of 3',5'-diiodothyronine (K_i 10 μM, D. Fekkes, E. van Overmeeren and T.J. Visser, unpublished data).

The experiments involving *N*-ethylmaleimide were also intended to give an answer to the question concerning the substrate specificity of the 5'-deiodinase. If the enzyme-sulphydryl group actively involved in the deiodination process, were the only one exposed to the environment, one would anticipate that occupation of the active site with substrate would prevent *N*-ethylmaleimide from blocking this group. In that case it would be possible to test by an entirely different approach which iodothyronines are substrates for a common enzyme.

The results once again demonstrate that thyroid hormone-deiodinating enzymes contain one or more essential cysteine residues. The failure of 3',5'-diiodothyronine to protect against the inactivation by *N*-ethylmaleimide may suggest that the catalytic thiol group is not shielded sufficiently by the substrate under the conditions tested. It may also indicate that essential sulphydryl groups are also located outside the active site of the enzyme.

In conclusion, the present paper demonstrates that 3,5-diiodothyronine is deiodinated by the enzyme catalysing the 5'-deiodination of other iodothyronines. The results at least indicate that thyroid hormone-deiodinating enzymes do not display full specificity with respect to the position of the iodine atoms in the substrate.

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