A Possible Function of Thiols, Including Glutathione, as Cofactors in the Conversion of Thyroxine to 3,3',5-Triiodothyronine in Rat Liver Microsomes

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Abstract

Thyroxine (T$_4$) is known to be converted to triiodothyronine (T$_3$) in rat liver microsomes. An endogenous stimulatory factor for the conversion of T$_4$ to T$_3$ was identified as reduced glutathione (GSH) by Sephadex G-15 gel chromatography of rat liver extract. The production of T$_3$ in rat liver microsomes was maximally stimulated above a GSH concentration of 4 mM, and an approximately 5-fold stimulation was attained. This degree of stimulation was approximately 10 times less than that observed with dithiothreitol (DTT).

Activity in the conversion of T$_4$ to T$_3$ was solubilized from the microsomes using 0.1% deoxycholate. When the solubilized microsomal preparation, pre-treated with DTT or GSH, was subjected to gel filtration on a Sepharose CL-6B column to separate the preparation from the thiol, almost no T$_3$ producing activity was observed in any of the collected fractions. However, when DTT or GSH was added to the fractions containing high molecular weight components, the activity of the production of T$_3$ was sustained. This finding indicates that these thiols play an essential role in the conversion of T$_4$ to T$_3$, possibly acting as cofactors.

Visser et al., (1976) reported that the production of T$_3$ was localized in the microsomal fraction of rat liver, and that it was enhanced by certain thiol compounds such as dithiothreitol (DTT) and 2-mercaptoethanol.

One of the functions of these thiols may be the stabilization of the sulfhydryl group(s) in the putative enzyme (Visser et al., 1976; Chopra, 1978). Another proposed function of thiols is to act as cofactors in the enzymic deiodination of T$_4$ (Visser et al., 1978; Balsam and Ingbar, 1978; Harris et al., 1979). However, evidence for the latter function is lacking. We have investigated the role of thiol in 5'-deiodination of T$_4$ in rat liver microsomes, focusing attention on reduced glutathione (GSH) which is the most abundantly present thiol compound in
tissues. The results indicated that thiols, including GSH, play an essential role in the conversion of T₄ to T₃, possibly acting as cofactors. Preliminary accounts of this work were presented at the meeting of the Japan Endocrine Society (Imai et al., 1979).

Materials and Methods

Materials
L-(¹²⁵I) T₃ was obtained from Dainabot Radioisotope Laboratories, Tokyo; L-T₄, L-T₃, GSH, iodoacetamide, and N-ethylmaleimide were purchased from Sigma Chemical Company, St. Louis, Mo.; DTT from Boehringer, Mannheim, sodium deoxycholate from Difco Laboratories, Detroit, Mich.; and 5,5-dithiobis-(2-nitrobenzoic acid) from Nakarai Chemicals, Kyoto. Sephadex G-15, Sephadex G-25 (fine), and Sepharose CL-6B were the products of Pharmacia Fine Chemicals, Uppsala. The antiserum used in RIA for T₃ was raised in rabbits as described by Mitsuma et al. (1971). T₄ was purified by column chromatography on Sephadex G-25 (Green, 1972). The T₄ obtained showed a relative displacement potency of less than 0.02% in the RIA as compared with T₃. The cross-reactivity of the antiserum with 3,3',5-triiodothyroacetic acid was 23%. The infraassay error of this assay was 6.1%, and the interassay error was 13.6%. The lower limit of the assay was 15.6 pg of T₃.

Preparation of rat liver microsomes
Male Wister rats weighing 150-200 g were used in the preparation of the microsomes. The rats were sacrificed by exanguination from the aorta under ether anaesthesia. Livers were homogenized at 0°C in 2.5 volumes of 0.25 M sucrose containing 20 mM Tris-HCl buffer (pH 7.4) and 1 mM EDTA. The homogenate was centrifuged at 10,000 g for 15 min, then the resulting supernatant was centrifuged at 105,000 g for 60 min at 4°C. The precipitated microsomes were stored at -20°C and used within 2 weeks.

Assay for the conversion of T₄ to T₃
The assay mixture, in a final volume of 1 ml, contained T₄ (250 ng), EDTA (1 μmol), and microsomes (approx. 2 mg of protein) in 20 mM Tris-HCl buffer (pH 7.4). The reaction was initiated by the addition of T₄, and the mixture was incubated in air for 30 min at 37°C. After being cooled in an ice bath, it was mixed with 1 ml of 0.1 N NaOH, and the T₃ produced was assayed by RIA as described by Surks et al., (1972). The control reaction mixture contained the same compounds as the sample except for the omission of microsomes.

Gel chromatography in the detection of a stimulatory factor in the production of T₃
Homogenate of rat liver (10 g) was prepared as described above, and a solution of 1/10 volume of 55% trichloroacetic acid (TCA) was added. The precipitate formed was removed by centrifugation at 4°C, and the TCA in the supernatant was extracted with ethyl ether. The volume of the solution was brought to 2 ml by lyophilization, and 1.2 ml of this sample was placed on a column of Sephadex G-15 (1.2 x 60 cm) which had already been equilibrated with 10 mM sodium acetate buffer (pH 4.0). The column was eluted with the same buffer at a flow rate of 21 ml/hr at 4°C, and 1.85 ml fractions were collected.

One-half milliliter of each fraction was neutralized with NaOH, and then incubated with rat liver microsomes (2.8 mg of protein) in the presence of T₄ (250 ng) in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA for 30 min at 37°C. The final volume of the reaction mixture was 1.0 ml. The T₃ produced was assayed as described above. Thiol contents in the fractions were measured as described by the Ellman (1959).

Solubilization of T₄ 5'-deiodinase from rat liver microsomes
Rat liver microsomes were solubilized at a concentration of 2 mg of protein per ml in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, utilizing various concentrations of deoxycholate (0.05-0.8%). After standing for 30 min at 0°C, the mixture was centrifuged at 105,000 x g for 60 min at 4°C. The supernatant was assayed for the conversion of T₄ to T₃ as described above. One millimolar concentration of DTT was included in the reaction mixture in this assay.

Effect of thiol treatment on T₄ 5'-deiodinase activity
T₄ 5'-deiodinase was solubilized from rat liver microsomes with 0.1% deoxycholate. This enzyme preparation was mixed with 1 mM DTT in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. After standing for 30 min at 0°C, the mixture was placed on a column of Sepharose CL-6B (1.4 x 65 cm) which had previously been equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. The column was eluted with the same buffer at a flow rate of 28 ml/hr at 4°C. Fractions of 1.85 ml were collected, and 0.5 ml of each fraction was assayed for activity of the conversion of T₄ to T₃ in the presence and absence of 1 mM DTT or 5 mM GSH.

Determination of protein
Protein was assayed as described by Lowry et al., (1951), using bovine serum albumin as the standard.
Results

The conversion of T$_4$ to T$_3$ in rat liver microsomes is known to be stimulated by thiol compounds (Visser et al., 1976; Chopra, 1978), and it was suspected that rat liver possesses an endogenous factor stimulating this conversion, and that GSH may be one such factor (Chopra, 1978; Kaplan, 1979). However, there has been no direct demonstration that GSH is a major, endogenous stimulatory factor. We attempted to show this by chromatography of rat liver extract. The factor could be detected by the enhanced production of T$_3$ when rat liver microsomes were incubated with T$_4$. Rat liver extract which is soluble in 5% TCA was subjected to gel chromatography on a Sephadex G-15 column. A stimulatory factor for the production of T$_3$ was found to be eluted at the same position where a thiol substance was eluted (Fig. 1). The elution position of this thiol corresponded to that of the authentic GSH, suggesting that the endogenous GSH is responsible for the stimulation of T$_3$ production. Although most of the TCA used for the extraction of the stimulatory factor was removed by extraction with ethyl ether, the remaining TCA, which was eluted after GSH, may have suppressed the production of T$_3$ as shown in Fig 1. The production of T$_3$ was also inhibited by the fractions (fraction 15–20) which were eluted between the void volume of the column and the GSH peak. This may indicate that rat liver contains an endogenous inhibitor for the 5'-deiodination of T$_4$.

The effect of varying concentrations of GSH on the microsomal conversion of T$_4$ to T$_3$ was examined and compared with the effect of DTT, which was reported to potentially stimulate this conversion. Three-tenth nanogram of T$_3$ was produced when T$_4$ (250 ng) was incubated with rat liver microsomes (2.1 mg of protein) in 1 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA for 30 min at 37°C. This production was found to be enhanced approximately 5-fold by the addition of 4 mM GSH. Figure 2A shows the rate of production of T$_3$ as a function of the concentration of GSH.

Fig. 1. Detection of a stimulatory factor for the 5'-deiodination of T$_4$ in rat liver. Protein of rat liver homogenate was precipitated with 5% TCA, and the resulting liver extract was subjected to gel chromatography on Sephadex G-15. Fractions of 1.85 ml were collected. 0.5 ml of each fraction was incubated with rat liver microsomes and T$_4$ in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA in the final volume of 1 ml for 30 min at 37°C. The T$_3$ produced (●) was assayed as described in the text. The concentration of thiol (○) in each fraction was measured by the method of Ellman (1959).
Fig. 2. The effect of GSH and DTT on the conversion of T₄ to T₃ with rat liver microsomes. A, T₄ (250 ng) was incubated with the microsomes (2.1 mg) in 1 ml of 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and the varying concentrations of GSH (0.1-8.0 mM) for 30 min at 37°C. The T₃ produced was assayed by RIA as described by Surks et al. (1973). B, The conditions were the same as in A except that DTT (0.125-5.0 mM) replaced GSH. Each point represents the mean ± SEM (n=3).

Table 1. The effect of sulphydryl reagents on the conversion of T₄ to T₃

<table>
<thead>
<tr>
<th>Sulphydryl reagent</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Iodoacetamide</td>
<td>96.6 ± 0.2</td>
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<tr>
<td>0.1 mM</td>
<td>96.3 ± 0.5</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>54.9 ± 1.3</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>24.1 ± 1.8</td>
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Rat liver microsomes were incubated with the indicated concentrations of iodoacetamide and N-ethylmaleimide for 15 min at 37°C, and then the activity of 5′-deiodination of T₄ was assayed in the presence of 2 mM DTT as described in the text. The values represent means ± SEM (n=3).

The rate leveled off above a GSH concentration of 4 mM, and half-maximal stimulation for the T₃ production was achieved at 850 μM. Still greater stimulation (ca. 50-fold) was observed with DTT as shown in Fig. 2B, and half-maximal stimulation with DTT was achieved at 90 μM, which was approximately 9 times lower than that observed with GSH. The concentration of DTT at which the production of T₃ was saturated (1 mM) was also lower than that of GSH (4 mM).

In order to determine whether the putative enzyme catalyzing the conversion of T₄ to T₃ was inactivated by blocking its sulphydryl group(s), the effect of sulphydryl reagents on the microsomal 5′-deiodination of T₄ was examined. Iodoacetamide almost completely inhibited the activity at 0.1 mM, and N-ethylmaleimide by 55% at 1 mM (Table 1). These results indicate that T₄ 5′-deiodinase has sulphydryl group(s) which may be essential for activity.

To further study the conversion of T₄ to T₃ catalyzed by the deiodinase, we attempted to solubilize the enzyme from rat liver microsomes. When deoxycholate (0.1%) was added to the suspension of the microsomes (2 mg protein/ml), approximately 30% of activity was found in the supernatant obtained by centrifugation at 105,000 × g for 60 min. A further increase in the concentration of deoxycholate decreased the yield of activity in the supernatant (Fig. 3). It appears that the enzyme is inactivated by a high concentration of the detergent. We tested the possibility of thiols serving as cofactors in the conversion of T₄ to T₃ by using the microsomal extract obtained with 0.1% deoxycholate. The extract was incubated with 1 mM DTT, after which high molecular weight components were separated from DTT by gel filtration on a Sepharose CL-6B column. If the thiols function only as stabilizing agents for sulphydryl group(s) of the enzyme, the enzyme pre-treated with the thiols should have retained its activity.
even after the enzyme was separated from the thiols by the gel filtration. However it was found that the enzyme alone contributed only marginal activity in the conversion of T₄ to T₃ when it was incubated with T₄. However, when DTT or GSH was added to the collected fractions containing the enzyme, there appeared substantial activity, as shown in Fig. 4. The activity with DTT was approximately 6 times greater than that with GSH. These results suggested that DTT and GSH act as cofactors in the 5'-deiodination of T₄ catalyzed by the enzyme. Figure 4 also shows that nearly 30% of the activity was eluted at the void volume of the column, and that the rest of the activity was eluted in the vast range of fractions (MW<4×10⁶). This indicates that most of the enzyme in the supernatant after centrifugation at 105,000×g for 60 min was really solubilized by the detergent. The elution patterns of activity observed with DTT and GSH were essentially the same, indicating that an identical enzyme participated in the conversion of T₄ to T₃, depending on which thiol was used as the stimulatory factor. When GSH (4 mM) was used in place of DTT in the pre-treatment of the enzyme, similar results were obtained.

Discussion

Recent studies have shown that various thiol compounds enhance the conversion of T₄ to T₃ in rat liver homogenate (Chopra, 1978), and in the microsomal fraction of the liver (Visser et al., 1976). There has been no detailed study on the effect of GSH on the 5'-deiodination of T₄ although among the naturally occurring thiol compounds GSH is most abundantly present in tissues. In the present study, a major endogenous factor stimulating the microsomal production of T₃ from T₄ was identified as GSH by gel chromatography of rat liver extract.²) It is noted in this regard that diamide, a relatively specific inactivator of GSH, diminished the rate of production of T₃ from T₄ in rat liver homogenate (Kaplan, 1979). It was also found that exogenous GSH substantially stimulated the conversion of T₄ to T₃ with rat liver microsomes, and that the rate of conversion was saturated at the physiological concentration of GSH, viz., 7.4 mM in rat liver (Davidson and Hird, 1964). It was reported that only slight stimulation was observed with rat liver homogenate (Chopra, 1978). The concentration of GSH at which the rate of production of T₃ was saturated (4 mM) was less than 2) The rate of production of T₃ with the fraction giving the highest activity (fraction 24) was 0.87 ng T₃/30 min/mg of protein, while the concentration of the thiol from the same fraction in the incubation mixture was 2.0 mM. It is noted that this rate of T₃ production is comparable to what is expected from the results shown in Fig. 2, B (0.66 ng T₃/30 min/mg of protein).
Fig. 4. The elution profile of gel chromatography on Sepharose CL-6B of the solubilized T₄ 5'-deiodinase. The enzyme was solubilized from rat liver microsomes with 0.1% deoxycholate, and treated with 1 mM DTT for 30 min at 0°C. The sample was subjected to gel filtration on Sepharose CL-6B at 4°C. Fractions were assayed for T₄ to T₃ conversion activity in the presence of 1 mM DTT (●) or 5 mM GSH (○) and in the absence of thiol (△), as described in the text. The fractions containing activity were well separated from those containing DTT: the peak of DTT was at fraction 54.

that of GSH in rat liver. It seems, therefore, that the lack of a stimulatory effect following the addition of GSH to the homogenate may be explained in terms of the presence of a nearly saturating concentration of GSH in the homogenate.

Both iodoacetamide and N-ethylmaleimide inhibited the enzymic conversion of T₄ to T₃ in rat liver microsomes. A similar inhibitory effect by sulfhydryl reagents such as p-chloromercuriphenylsulfate and Hg⁺ was reported previously (Visser, 1976). These findings suggest that the putative enzyme possesses sulfhydryl group(s) which may be essential for its activity. Accordingly, it is conceivable that thiols have the effect of stabilizing the sulfhydryl group(s) of the enzyme.

One important aspect of the present study is the demonstration that thiols are required for the enzyme to manifest its activity. The solubilized enzyme, pre-treated with DTT or GSH, was almost inactive when it was separated from these thiols by
gel chromatography. The addition of the thiols to the collected fraction containing the enzyme, however, resulted in substantial activity. Since the separation of the enzyme from the thiols was made at 4°C within 3 hr, the inactivation of the enzyme due to oxidation of its sulfhydryl group(s) during chromatography may be unlikely. Thus, this finding suggests that the thiols serve as cofactors in the enzymic 5'-deiodination of T₄. Alternatively, the essential sulfhydryl group(s) in the enzyme may be prone to oxidation. If this oxidation does occur, its velocity would be fairly fast. In this case, the enzyme would be inactive without coexisting thiol compounds. At any rate, it became clear from the present study that thiols, including GSH, play an essential role in the enzymic conversion of T₄ to T₃.

References
