Binding of Radioiodinated Propylthiouracil to Rat Liver Microsomal Fractions

STIMULATION BY SUBSTRATES FOR IODOTHYRONINE 5'-DEIODINASE

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Previous studies have shown that 2-thiouracil derivatives are uncompetitive inhibitors of iodothyronine 5'-deiodinase activity of rat liver microsomal fraction. Therefore the interaction of radioiodinated 6-propyl-2-thiouracil with rat liver microsomal fraction and the effect of substrate, cofactor and other inhibitors of 5'-deiodinase activity were investigated. It was found that micromolar concentrations of, in order of increasing potency, 3,5-diiodotyrosine, thyroxine, 3,3',5'-tri-iodothyronine and 3',5'-di-iodothyronine significantly enhanced binding of 5-[¹²⁵I]iodo-6-propyl-2-thiouracil to the enzyme preparation. This stimulation was not seen in the presence of 1 mM-dithiothreitol, 0.1 mM-6-propyl-2-thiouracil, 0.1 M-2-mercapto-1-methylimidazole or 1 mM-sodium sulphite. These results support the hypothesis that thiouracil derivatives inhibit 5'-deiodinase activity by forming a mixed disulphide with an intermediate enzyme complex, probably a sulphenyl iodide.

Investigations by Chopra and co-workers (Chopra, 1977; Chopra *et al.*, 1978), by Leonard & Rosenberg (1978) and by one of us (Visser, 1979a) have demonstrated that 2-thiouracil derivatives are uncompetitive inhibitors of iodothyronine 5'-deiodinase. Enzymic 5'-deiodination follows a Ping Pong mechanism with thiol compounds as second substrate (cofactor) (Leonard & Rosenberg, 1978; Visser, 1979a). Inhibition by thiouracil is competitive with cofactor (Leonard & Rosenberg, 1978; Visser 1979a). Thyroid hormone-deiodinating enzymes contain essential thiol groups (Visser, 1978). Thiouracil is regarded as a selective reagent for sulphenyl iodide groups forming mixed disulphides (Cunningham, 1964). It was therefore proposed (Visser, 1979a) that 5'-deiodination occurs as follows:

where TI_n and TI_{n-1} are substrate and product iodothyronines respectively.

To prove the actual formation of the E-SI intermediate may not be possible, owing to its lability in aqueous solutions (Cunningham, 1964). If the proposed pathway is correct, however, one should be able to observe the formation of a mixed disulphide on reaction of the iodo-enzyme complex with radioactive thiouracil. In the present paper we report that binding of a radioiodinated thiouracil derivative (5-[¹²⁵I]iodo-6-propyl-2-thiouracil) to rat liver microsomal fraction is induced by substrates of iodothyronine 5'-deiodinase, e.g. 3,3',5'-tri-iodothyronine ('reverse tri-iodothyronine').

Materials and Methods

Preparation of 5-[¹²⁵I]iodo-6-propyl-2-thiouracil

6-Propyl-2-thiouracil, obtained by the courtesy of Dr. P. J. H. Eggels, Academic Hospital 'Dijkzigt', Rotterdam, The Netherlands, was radioiodinated by using chloramine- τ and carrier-free ¹²⁵I⁻ (The Radiochemical Centre, Amersham, Bucks., U.K.) in 0.1 M-sodium acetate, pH 3.3 (e.g. Hadi *et al.*, 1978). At this low pH, oxidation of the sulphur does not occur (R. H. Lindsay, personal communication). The reaction mixture was purified by adsorption chromatography on a small Sephadex G-10 column with 0.1 M-acetic acid as the eluent. This resulted in a clear separation of 5-[¹²⁵I]iodo-6-propyl-2-thiouracil from 6-propyl-2-thiouracil and ¹²⁵I⁻. On the basis of the specific radioactivity of ¹²⁵I⁻ given by the supplier (approx. 14 mCi/ μ g), the specific radioactivity of the product was calculated to be approx. 6 mCi/ μ g (1750 Ci/mmol).

Binding of 5-[¹²⁵I]iodo-6-propyl-2-thiouracil to rat liver microsomal fraction

Rat liver microsomal fraction in 0.05 M-sodium phosphate/3 mM-EDTA/0.1 mM-dithiothreitol, pH 6.5, was prepared essentially as previously described (Visser *et al.*, 1976). Microsomal protein (2 mg) was incubated for 30 min at 37°C with 1 μ Ci of 5-[¹²⁵I]iodo-6-propyl-2-thiouracil (approx. 5 pmol) in the presence of various concentrations of reverse tri-iodothyronine in 1 ml of 0.05 M-sodium phosphate/3 mM-EDTA, pH 6.5. The final dithiothreitol con-

centration was 0.01 mM. The reaction was stopped and microsomal protein was precipitated by the addition of 2 ml of acetone at 0°C. After centrifugation for 10 min at 1500g and aspiration of the supernatant, the pellets were usually dissolved in 1% sodium dodecyl sulphate by treatment for 1 h at 60°C and protein was again precipitated with 2 ml of acetone at 0°C. Radioactivity in the pellets was measured (in a Searle gamma spectrometer) with an efficiency of 85%. Microsomal protein was measured by using the method of Spector (1978) with bovine serum albumin as a standard.

Results

If the reaction was carried out in the absence of reverse tri-iodothyronine, only approx. 0.7% of added 5-[¹²⁵I]iodo-6-propyl-2-thiouracil was precipitated with acetone. After washing by solubilization of the pellet in 1% sodium dodecyl sulphate followed by another precipitation with acetone the radioactivity associated with the protein decreased to 0.2–0.3%. If microsomal protein was precipitated with 10% trichloroacetic acid approx. 40% of radioactivity was spun down. After solubilization of the pellet in 0.5M-NaOH and precipitation with 10% trichloroacetic acid the radioactivity remaining in the precipitate decreased to approx. 8.5%. Repetition of this procedure resulted in a further decrease to approx. 2.5%.

Fig. 1 demonstrates that the binding of radioactive thiouracil was greatly enhanced by co-incubation with reverse tri-iodothyronine. Addition of 0.1 μM-

Fig. 1. Dependence of binding of radioactive propylthiouracil to rat liver microsomal fraction on reverse tri-iodothyronine concentration

Precipitated radioactivity before (●) and after (○) washing was measured as described in the Materials and Methods section. Results are means ± S.D. for five measurements.

substrate resulted in a significant stimulation of binding. The increment in protein-bound radioactivity remained virtually the same after washing with 1% sodium dodecyl sulphate. The effect of reverse tri-iodothyronine appeared to reach a plateau at substrate concentrations above 10 μM. Table 1 demonstrates that the association of 5-[¹²⁵I]iodo-6-propyl-2-thiouracil with the protein as induced by optimal concentrations of reverse tri-iodothyronine was prevented by addition of 1 mM-dithiothreitol, 100 μM-6-propyl-2-thiouracil, 100 mM-2-mercapto-1-methylimidazole (methimazole) or 1 mM-sodium sulphite. The addition of these compounds also decreased the binding of radioactive thiouracil in the absence of reverse tri-iodothyronine. Binding was decreased from 42% to 27% by the addition of 20 μM-6-propyl-2-thiouracil and to 20% by 1 mM-dithiothreitol as analysed by precipitation with trichloroacetic acid. By using this method no significant stimulation by reverse tri-iodothyronine could be detected.

It was found that 3',5'-di-iodothyronine was more effective than reverse tri-iodothyronine in stimulating binding of radioiodinated 6-propyl-2-thiouracil to the microsomal fraction, whereas thyroxine and 3,5-di-iodotyrosine were less effective (Table 1).

Discussion

A related study was published in abstract form recently by Chopra and his co-workers (Yamada *et al.*, 1978). One important difference from the present study is the use by these authors of commercially available ³⁵S-labelled propylthiouracil of low specific radioactivity (approx. 20 mCi/mmol) compared with the ¹²⁵I-labelled derivative (approx. 1750 Ci/mmol). Another major difference is that these workers used rat liver supernatant fraction, containing very little enzyme activity, which is most probably accounted for by contamination with microsomal fraction (Fekkes *et al.*, 1979). Nevertheless, they assumed that binding of radioactive thiouracil to iodothyronine 5'-deiodinase was studied, which was inhibited by reduced glutathione, but not affected by thyroxine, another substrate of this enzyme. Since these investigators themselves previously reported on the uncompetitive inhibition by propylthiouracil of 5'-deiodination (Chopra 1977; Chopra *et al.*, 1978) one would anticipate reaction with the inhibitor to occur only if conditions for deiodination are fulfilled, i.e. in the presence of substrate. Similar findings to those of Yamada *et al.* (1978) were obtained if microsomal protein was precipitated with trichloroacetic acid (see the Results section). Similar observations were also made with this method if bovine serum albumin instead of microsomal protein was used (T. J. Visser and E. van Overmeeren, unpublished work). Only if non-specific binding of the lipophilic propyl-

Table 1. Stimulation of binding of 5-[¹²⁵I]iodo-6-propyl-2-thiouracil to rat liver microsomal fraction by iodoamino acids and inhibition by affectors of iodothyronine 5'-deiodinase activity

Statistical testing of stimulation by iodoamino acids (Student's *t* test): *not significant; †*P* < 0.001. For experimental details see the Materials and Methods section. Results are means ± S.D. (*n* = 4).

Expt. 1	Iodoamino acid (10 μM)	Test substance	Binding of radioactive label (%)	
			Before washing	After washing
—	—	—	2.14 ± 0.03	0.37 ± 0.01
3,3',5'-Tri-iodothyronine	—	—	2.77 ± 0.17†	0.72 ± 0.09†
3,3',5,5'-Tetraiodothyronine (thyroxine)	—	—	2.52 ± 0.08†	0.55 ± 0.05†
3',5'-Di-iodothyronine	—	—	3.10 ± 0.24†	0.91 ± 0.15†
3,5-Di-iodotyrosine	—	—	2.41 ± 0.07†	0.55 ± 0.03†
Expt. 2	—	—	2.9 ± 0.2	0.90 ± 0.04
3,3',5'-Tri-iodothyronine	—	—	6.9 ± 0.6†	2.65 ± 0.36†
—	—	1 mM-Dithiothreitol	2.0 ± 0.2	0.25 ± 0.01
3,3',5'-Tri-iodothyronine	—	1 mM-Dithiothreitol	2.0 ± 0.2*	0.25 ± 0.01*
—	—	0.1 mM-6-Propyl-2-thiouracil	2.2 ± 0.2	0.41 ± 0.04
3,3',5'-Tri-iodothyronine	—	0.1 mM-6-Propyl-2-thiouracil	2.0 ± 0.2*	0.40 ± 0.02*
—	—	0.1 M-2-Mercapto-1-methylimidazole	2.2 ± 0.2	0.23 ± 0.01
3,3',5'-Tri-iodothyronine	—	0.1 M-2-Mercapto-1-methylimidazole	2.0 ± 0.1*	0.24 ± 0.02*
—	—	1 mM-Sodium sulphite	2.2 ± 0.1	0.44 ± 0.03
3,3',5'-Tri-iodothyronine	—	1 mM-Sodium sulphite	2.1 ± 0.1*	0.44 ± 0.01*

thiouracil is disposed of by precipitation with acetone and radioactively labelled inhibitor of high specific radioactivity is used, substrate-induced binding may be observed.

Although no direct proof was obtained for binding of thiouracil to the 5'-deiodinase, there is considerable circumstantial evidence that this is the case. Compounds known to influence deiodinase activity by reacting with the sulphenyl group of the intermediate enzyme complex (Leonard & Rosenberg, 1978; Visser, 1979a,b) were found to inhibit thiouracil incorporation (Table 1). Furthermore, it was shown that induction by reverse tri-iodothyronine is a saturable process suggesting the involvement of a limited number of high-affinity binding sites. The concentration of reverse tri-iodothyronine needed to exert a significant enhancement of thiouracil incorporation was substantially higher than the K_m of this substrate for the 5'-deiodinase, i.e. 0.035 μM (Visser *et al.*, 1979). This probably results from the high concentration of microsomal fraction employed, containing a large number of non-specific binding sites for iodothyronines (for discussion see Visser *et al.*, 1978, 1979). Finally, the relative potency of the various iodoamino acids in stimulating thiouracil incorporation (Table 1) mirrored their performance as substrates for iodothyronine 5'-deiodinase activity (Visser *et al.*, 1979; T. J. Visser & E. van Overmeeren, unpublished work).

In conclusion, the present paper reports circumstantial evidence for the covalent attachment of radioiodinated propylthiouracil to an intermediate enzyme complex that is formed during 5'-deiodin-

ation. Much more study is required to actually prove the proposed sequence of events. An important practical consequence of the selective labelling of the 5'-deiodinase with radioactive thiouracil is that it may provide a valuable tool in the purification of the enzyme.

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