Structural requirements of thiol compounds in the inhibition of human liver iodothyronine 5'-deiodinase

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2-Thiouracil and a number of its alkyl derivatives are known to inhibit the enzymic 5'-deiodination of thyroxine to 3,5,3'-tri-iodothyronine. The structural requirements for inhibition of iodothyronine 5'-deiodinase were investigated by using a washed postmitochondrial particulate fraction of human liver. A series of sulphur-containing derivatives of pyrimidine, pyridine, imidazole, benzene and urea, capable of existing in a thiol form, were incubated at several concentrations with the enzyme preparation in the presence of thyroxine and dithioerythritol (cofactor). The degree of inhibition by the respective compounds of the production of 3,5,3'-tri-iodothyronine was studied in relation to their structural features. The major observations were: (i) a free thiol group is essential; (ii) compounds that do not possess a polar hydrogen atom spatially configured so that it is proximal to the thiol group are poor inhibitors; (iii) aromatic characteristics in the presence of requirements (i) and (ii) lead to the expression of potent inhibitory properties; (iv) modification of potent inhibitors by the introduction of hydrophilic substituents reduces the inhibitory potency.

Extra-thyroidal production of 3,5,3'-tri-iodothyronine in liver and kidney by 5'-deiodination of thyroxine has been established as the major source of biologically active thyroid hormone (Hershman, 1964; Braverman *et al.*, 1970; Pittman *et al.*, 1971; Visser *et al.*, 1975). The enzyme catalysing this reaction, iodothyronine 5'-deiodinase, prepared from rat liver and kidney, has been reported to be microsomal in origin (Hesch *et al.*, 1975; Visser *et al.*, 1976; Leonard & Rosenberg, 1978) and to possess an essential thiol group (Visser *et al.*, 1976; Chopra, 1977). Thiol compounds are required to maintain enzyme activity, and dithiothreitol is the most effective thiol in 'in vitro' systems (Visser *et al.*, 1976; Chopra, 1978).

Thyroid peroxidase inhibitors, including 2-thiouracil and 6-n-propyl-2-thiouracil (Taurog, 1976), have been shown to be potent inhibitors of 5'-deiodination *in vivo* (Hershman, 1964; Geffner *et al.*, 1975) and *in vitro* (Visser & van Overmeeren, 1979; Visser *et al.*, 1979; Chopra *et al.*, 1982). These compounds react with protein sulphenyl iodides as in thyroid peroxidase to form protein-thiouracil mixed disulphides (Cunningham, 1964; Jirousek, 1968; Taurog, 1976), and it has been proposed that deiodination of thyroxine probably occurs also through a sulphenyl iodide intermediate (Leonard & Rosenberg, 1978; Visser, 1980). Visser

(1980, 1981) and Leonard & Rosenberg (1978, 1980) described a Ping Pong mechanism in which, after deiodination of thyroxine and release of 3,5,3'-tri-iodothyronine, the sulphenyl iodide intermediate is reduced by thiol cofactor to regenerate the active form of the enzyme. In the presence of 2-thiouracil the sulphenyl iodide reacts to form an inactive enzyme-thiouracil mixed disulphide, which may be subsequently reduced by cofactor. The more potent inhibitors of thyroid peroxidase, thiourea and *N*-methyl-2-mercaptoimidazole, do not inhibit 5'-deiodination (Visser *et al.*, 1979; Visser, 1980). These compounds have been shown to obviate the inhibition of 5'-deiodination by 2-thiouracil (Leonard & Rosenberg, 1978, 1980), and it has been suggested that, although they react rapidly with the sulphenyl iodide, the disulphide formed is unstable and readily reduced to the active enzyme by dithiothreitol (Visser, 1980). Thyroid peroxidase is inhibited by most thioamides, including 2-thiouracil (Taurog, 1976), and these inhibitory properties have been associated with the ability of the thioamide to exist in a thiol form (Cunningham, 1964; Taurog, 1976).

Previous studies of iodothyronine 5'-deiodinase inhibitors (Hershman & Van Middleworth, 1962; Visser *et al.*, 1979; Chopra *et al.*, 1982) have indicated that the inhibition of this enzyme, unlike that

of thyroid peroxidase, requires other structural factors in addition to the existence of a thiol tautomer. These postulated requirements include the thioureylene structure $[-NH-C(=S)-NH-]$, an oxygen atom at the 4-position of 2-thiopyrimidines, and a secondary amine as the N^1 nitrogen of 2-thiopyrimidines and 2-mercaptoimidazoles. We have investigated the inhibitory properties of a number of sulphur-containing compounds, including those with the thioureylene structure, in an attempt to elucidate the structural requirements for inhibition of human liver iodothyronine 5'-deiodinase.

Materials and methods

Materials

Thyroxine and 3,5,3'-tri-iodothyronine, of high purity, were obtained from Calbiochem-Behring Corp., CP Laboratories, Bishop's Stortford, Herts., U.K. Phenol and thiophenol were obtained from BDH, Poole, Dorset, U.K. 2-Imidazolidinethione, 2-mercaptoimidazole and *N*-methylthiourea were obtained from Lancaster Synthesis, Morecambe, Lancs., U.K. 2-Mercaptophenol and 3-mercaptophenol were synthesized in this laboratory by the method of Djerassi *et al.* (1954). *N*¹-methyl-6-methyl-2-thiouracil was synthesized from *N*-methylthiourea and ethyl acetoacetate (Fitton & Smalley, 1968). The structures and purities of the preparations were verified by spectroscopic studies and t.l.c. All other thiols were obtained from the Aldrich Chemical Co., Gillingham, Dorset, U.K. Specimens of liver were obtained from fatal-road-traffic-accident subjects, permission of the next-of-kin having been obtained.

Methods

Liver, free of large blood vessels, was washed in 0.05 M-sodium phosphate buffer, pH 7.5, containing 6 mM-EDTA and homogenized at 0°C in 2 vol. of this buffer containing 0.25 M-sucrose and 2 mM-dithioerythritol. Homogenates were filtered through polyester gauze and centrifuged at 2000 *g* for 15 min to remove nuclei and debris. Mitochondria were removed by centrifugation at 10000 *g* for 15 min at 4°C. Aggregation of the residual particulate material (mainly 'microsomes') was achieved by adjusting the pH of the supernatant to 6.5 with NaH_2PO_4 (1.0 M) and adding poly(ethylene glycol) 6000 to a final concentration of 0.02 g/ml. After being left for 30 min at 0°C the aggregated material was precipitated by centrifuging at 10000 *g* for 30 min. The pellet, containing 90% of the iodothyronine 5'-deiodinase activity present in the postmitochondrial supernatant, was

resuspended in 0.05 M sodium phosphate buffer, pH 6.8, containing 2 mM-dithioerythritol and again centrifuged. The supernatant was discarded and the pellet made up in a volume of the latter buffer equal to that of the liver used and stored at -20°C in 1.5 ml aliquots. The enzyme was stable under these conditions for 2 months.

The effect of the various compounds under investigation on 5'-deiodination of thyroxine was studied by incubating, at 37°C, 0.1 ml of enzyme preparation (containing 1.7 mg of protein/ml) with 10 μ M-thyroxine, 2 mM-dithioerythritol and various concentrations of the test material in a total volume of 2 ml of 0.05 M-phosphate buffer, pH 6.8. Where necessary, test substances were dissolved in a minimum volume of methanol followed by dilution in the pH 6.8 buffer. The maximum concentration of methanol present in the incubation mixture was 2%, and when included in control experiments it did not affect deiodinase activity. The reaction was initiated by the addition of enzyme preparation. Samples (0.2 ml) were taken at zero time and then at 15 min intervals up to 60 min. The samples were immediately added to 0.4 ml of 0.1% sodium dodecyl sulphate to stop the reaction. 3,5,3'-Tri-iodothyronine was determined in duplicate 20 μ l aliquots by a specific double-antibody radioimmunoassay procedure. Rates of formation of 3,5,3'-tri-iodothyronine were linear over the first 60 min. The zero-time sample contained less than 2 nmol of 3,5,3'-tri-iodothyronine/litre and the compounds under investigation did not alter this value. The formation of 3,5,3'-tri-iodothyronine in the absence of enzyme or dithioerythritol did not exceed 1.5% of that obtained for the activated enzyme. All thyroxine 5'-deiodinase assays were performed in duplicate and inter-assay precision was monitored by including four controls in each assay. These controls were: (a) complete incubation mixture with no inhibitor; (b) as (a) plus 6-n-propyl-2-thiouracil (4 μ M), giving approx. 50% inhibition; (c) as (a) plus 2-thiouracil (64 μ M), giving approx. 50% inhibition; and (d) with no dithioerythritol. The coefficient of variation ($n = 28$) for (a), (b) and (c) was less than 7.5% and for (d) 22%.

Results

The effect of increasing concentration of compounds containing the thioureylene structure on the 5'-deiodination of thyroxine to 3,5,3'-tri-iodothyronine is shown in Figs. 1 and 2. Of the 6-alkyl derivatives of 2-thiouracil investigated, a methyl substituent had no effect on inhibitory potency, whereas the propyl substituent increased activity 15.5 times. The presence of a benzo group, as in 2-

Symbol	Compound	Structure	Symbol	Compound	Structure
△	2-Thiouracil		▲	2-Mercaptopyrimidine	
▼	6-Methyl-2-thiouracil		○	2-Thiocytosine	
■	6-n-Propyl-2-thiouracil		◆	6-Amino-2-thiouracil	
●	2-Mercapto-4(3H)-quinazolinone		◇	5-Carboxy-2-thiouracil	
□	N ¹ -Methyl-6-methyl-2-thiouracil		▽	2-Mercaptopurine-6,8-dione	

Fig. 1. Comparison of the relative potencies of 2-mercaptopyrimidine derivatives in inhibiting the production of 3,5,3'-triiodothyronine from thyroxine by human liver iodothyronine 5'-deiodinase. Conditions were as described in the Materials and methods section. The substances tested are shown above.

mercapto-4(3H)-quinazolinone (benzo-[e]-2-thiouracil), produced a marked increase in inhibitory potency, the latter being approx. 100 times as active as the parent 2-thiouracil. This compound

was the most potent inhibitor tested in the present study. Similarly, the presence of a benzo group at the 4- and 5-positions of 2-mercaptoimidazole resulted in a large increase in potency; 2-mercapto-

Symbol	Compound	Structure	Symbol	Compound	Structure
Δ	Thiourea		Δ	N-Methyl-2-mercaptoimidazole	
Δ	N-Methylthiourea		●	2-Mercaptobenzimidazole	
○	2-Imidazolidinethione		□	2-Mercaptobenzoxazole	
■	2-Mercaptoimidazole		▽	2-Mercaptobenzothiazole	
▲	2-Thiohydantoin		Δ	2-(Methylmercapto)benzimidazole	

Fig. 2. Comparison of the relative potencies of thiourea and 2-mercaptoimidazole derivatives in inhibiting the production of 3,5,3'-tri-iodothyronine from thyroxine by human liver iodothyronine 5'-deiodinase

Conditions were as described in the Materials and methods section. The substances tested are shown above. Those identified with the same symbol (Δ) exhibited no inhibitory activity.

benzimidazole having 53 times the potency of the parent compound, and 14 times that of 2-thiouracil.

Introduction of hydrophilic substituents into the

5- and 6-positions of 2-thiouracil (5-carboxy-2-thiouracil, 6-amino-2-thiouracil and 2-mercaptapurine-6,8-dione), reduced inhibitory activity. Non-aromatic and acyclic compounds, although

Fig. 3. Comparison of the relative potencies of 2-mercaptopyridine and mercaptophenol derivatives in inhibiting the production of 3,5,3'-tri-iodothyronine from thyroxine by human liver iodothyronine 5'-deiodinase

Conditions were as described in the Materials and methods section. The substances tested are shown above.

possessing the thioureyne structure (2-thiohydantoin, 2-imidazolidinethione, thiourea and *N*-methylthiourea) proved to be very poor inhibitors. The most potent of these, 2-thiohydantoin, inhibited 5'-deiodinase only when present at high concentration (10mM).

Methylation of the sulphur of 2-mercaptobenzimidazole yields 2-(methylmercapto)benzimidazole, a compound with no observable inhibitory properties, demonstrating the requirement for a free thiol group in the inhibitory process. Replacement of the imino nitrogen of 2-mercaptobenzimidazole by oxygen or sulphur also gave rise to compounds with low inhibitory potency (2-mercaptobenzoxazole and 2-mercaptobenzothiazole). The presence of the *N*-methyl group in both *N*¹-methyl-6-methyl-2-thiouracil and *N*-methyl-2-mercaptoimidazole resulted in a loss of inhibitory activity. In contrast with 2-mercapto-

imidazole, 2-mercaptopyrimidine was without inhibitory activity. 4-Amino-2-thiopyrimidine (2-thiocytosine) was also inactive.

The effect of increasing the concentration of a further series of thiol-containing compounds on the 5'-deiodination of thyroxine is shown in Fig. 3. The most potent compound of this group, 2-mercapto-3-pyridinol, was 41 times as active as 2-thiouracil, whereas the non-hydroxylated compound, 2-mercaptopyridine, was inactive. The compound 2-mercaptophenol was a potent inhibitor, its potency being equal to that of 2-thiouracil, which in contrast with the *meta* isomer, 3-mercaptophenol, was almost non-inhibitory, as were thiophenol and phenol.

Discussion

A compound must be able to exist in the thiol form to react with a sulphenyl iodide intermediate to produce a mixed disulphide (Cunningham, 1964; Taurog, 1976) and the compounds investigated in the present study are, therefore, depicted as thiols. The results obtained show that, for compounds which undergo thione/thiol tautomerization, the relative stability of the two forms is not related to inhibitory potency. This is demonstrated by the observation that 2-thiouracil and 2-mercaptobenzimidazole, which exist predominantly as a thione and a thiol respectively at pH 6.8, are both potent inhibitors of iodothyronine 5'-deiodinase. Conversely, some thiones, e.g. 2-thiocytosine, and thiols, e.g. *N*-methyl-2-mercaptoimidazole, exhibit no inhibitory activity. However, although a thiol group is necessary for disulphide formation, other structural features determine the stability of the enzyme-inhibitor mixed disulphide.

The results and structures in Figs. 1 and 2 show that the potent inhibitors in these series possess a polar hydrogen atom and a thiol group on adjacent ring atoms. This appears to be essential for the expression of inhibitory activity, since modifications to the inhibitors that remove the polar hydrogen atom result in a marked loss of activity, as shown by the substitution of the polar *N*-hydrogen of 2-mercaptoimidazole by a methyl group and the replacement of the imino-ring nitrogen atom of 2-mercaptobenzimidazole by oxygen or sulphur. Comparison of the low inhibitory activity of 2-mercaptopyrimidine (Fig. 1), which does not have a polar hydrogen atom, with the high potency of 2-mercaptoimidazole (Fig. 2), which does, also supports the involvement of a polar hydrogen atom in the inhibition of iodothyronine 5'-deiodinase. Methylation at the N-1 position of 6-methyl-2-thiouracil restricts tautomerization of the thione/thiol to the N-3 hydrogen (Fig. 1) so that in the

thiol form there is no polar hydrogen atom on an adjacent ring atom, with a resulting loss of inhibitory activity. 2-Thiouracil may exist in an oxo or enol form, the polar hydrogen atom being present on the N-3 nitrogen in the former and on the C-4 oxygen in the latter. The requirement for the polar hydrogen atom and the thiol group to be on adjacent ring atoms is indicated by the low inhibitory potency of 2-thiocytosine, which although possessing polar hydrogen atoms as a C-4 amino substituent, has no polar hydrogen atom on the N-3 nitrogen. The suggestion that the absence of a C-4 oxygen atom in 2-thiocytosine is the cause of the low inhibitory activity (Visser *et al.*, 1979) seems unlikely, since both 2-mercaptoimidazole and 2-mercaptobenzimidazole, which are devoid of oxygen, are potent inhibitors. The high inhibitory activity of 2-mercapto-3-pyridinol and 2-mercaptophenol, which have a proximal polar hydrogen atom (phenolic OH *ortho* to the thiol), compared with the low activity of 3-mercaptophenol (Fig. 3), which possesses a more distal but equally polar hydrogen atom (phenolic OH *meta* to the thiol) demonstrates that inhibitory properties are dependent on the presence of a polar hydrogen atom proximal to the thiol group. The greater potency of 2-mercapto-3-pyridinol compared with 2-mercaptophenol might arise from the presence of the zwitterionic form of the former, in which the polar nature of the hydrogen atom is increased by the positively charged nitrogen atom. A previous claim (Visser *et al.*, 1979) that the thioureyne structure is essential for expression of inhibitory properties is not supported by these observations, which suggest that this structure is only a specific example of the general requirement for a polar hydrogen atom proximal to the thiol group. These findings are consistent with the formation of a hydrogen bond between enzyme and inhibitor and suggest that this is an important factor in stabilizing the enzyme-inhibitor mixed disulphide against reduction by cofactor.

The inactivity of thiourea and low inhibitory activity of 2-imidazolidinethione, which have a suitably configured polar hydrogen atom, demonstrates that additional structural features are necessary for the expression of inhibitory properties. The increase in inhibitory potency observed with the introduction of aromatic properties, i.e. 2-thiohydantoin to 2-thiouracil, and 2-imidazolidinethione to 2-mercaptoimidazole (Fig. 2), together with the demonstration of the increased activity of the benzo derivatives of 2-thiouracil and 2-mercaptoimidazole compared with the parent compounds, suggests that an aromatic nature is the other major structural feature which plays an important role in enhancing the stability of the enzyme-inhibitor mixed disulphide. It is observed

that a 6-n-propyl substituent also increases the inhibitory potency of 2-thiouracil, and this might result from its enhanced hydrophobic character. This need not discount the requirement for aromatic properties, since these are also regarded as being hydrophobic in nature. It is possible that greater steric exclusion of cofactor by the larger inhibitors [6-n-propyl-2-thiouracil, 2-mercapto-4(3*H*)-quinazolinone and 2-mercaptobenzimidazole] may give rise to the increased potency of these compounds. Derivatives of 2-thiouracil with hydrophilic substituents in the 5- and 6-positions (2-mercaptapurine-6,8-dione, 6-amino-2-thiouracil and 5-carboxy-2-thiouracil) have been shown to be less potent than 2-thiouracil. Since hydrophilic groups are also spatially restricting, the results are not wholly consistent with steric exclusion, and the requirement of aromatic or hydrophobic properties is a more probable explanation.

The inability of aromatic or hydrophobic properties to produce potent inhibitory activity in thiol compounds not possessing a polar hydrogen atom (2-mercaptobenzoxazole and 2-mercaptobenzothiazole) would suggest that the effect of the hydrophobic group is to stabilize the hydrogen bond between enzyme and inhibitor, possibly by preventing the access of water, which would readily break the bond and reduce inhibitory potency. The fact that hydrogen-bonding is more effective in a hydrophobic environment (cf. DNA) gives support to this suggestion.

The structural requirements of thiol inhibitors in determining the stability of the enzyme-inhibitor mixed disulphide have been elucidated with dithioerythritol as cofactor. However, the structure of the cofactor might also influence the stability of the enzyme-inhibitor mixed disulphide, and it is possible that different inhibitory potencies would be expressed in the presence of the, as yet unidentified, physiological cofactor.

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