Sites of Action of 2-Thiazoline-2-Thiol on Biogenesis of Thyroid Hormones

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ABSTRACT—2-Thiazoline-2-thiol is an antithyroid agent that strongly reduces thyroid hormone levels. Synthesis of these hormones is catalyzed in vivo by thyroid peroxidase. The interaction of this drug with molecular iodine and its effect on peroxidase activity were investigated. Iodine and 2-thiazoline-2-thiol form a complex of the charge transfer type of 1:1 stoichiometry characterized by a formation constant of 2,527 M⁻¹ at 20°C. This drug was found to inhibit both horseradish peroxidase and lactoperoxidase (used as a model of thyroid peroxidase) in a competitive manner, giving inhibition constants of 5.7 mM and 0.13 mM, respectively. T₃ and T₄ levels were reduced significantly after a three-week administration of this drug to a group of 10 rats. Histological examination of the thyroid gland showed the presence of a cylindrical epithelium, which is indicative of hyperactivity of the gland. The results indicated that 2-thiazoline-2-thiol acts on both molecular iodine and thyroid peroxidase.

Synthetic antithyroid agents are thought to exert their action on thyroid peroxidase, the enzyme responsible for the oxidation of iodides and the coupling of iodotyrosines to iodothyronines (1, 2). We showed that they may also interact with molecular iodine (3). Iodine is complexed by the drug, and thus becomes unavailable for synthesis of thyroid hormones. A drug which has activity towards TPO and which can complex molecular iodine will thus have particularly strong antithyroid activity. In the present study, we investigated the action of the antithyroid drug 2T2T (4), which has a partially hydrogenated thiazole nucleus. This drug also contains a thiol group, which is also found in MMI, a powerful anti-thyroid agent. We also tested action of 2T2T in vivo in the rat to find out whether the combined actions on peroxidase and iodine did in fact confer strong antithyroid activity.

MATERIALS AND METHODS

Chemicals

Iodine was from Merck (suprapur bisublimed ref. 4763, Darmstadt, Germany), and was used without further purification. It was kept in the dark in a dessicator containing P₂O₅. 2T2T was from Fluka (ref. 63860, Buchs, Switzerland) and was purified by HPLC (Waters, St. Quentin, Yvelines, France). Carbon tetrachloride was from Merck

Abbreviations used are: TPO, thyroid peroxidase; 2T2T, 2-thiazoline-2-thiol; MMI, 2-mercapto-1-methylimidazole; HRPO, horseradish peroxidase; LPO, lactoperoxidase; ABTS, 2,2' azino-bis-(3)-ethylbenzothiazolinesulfonic acid; BSB, blue-shifted band; CTB, charge-transfer band.
(Uvasol 2209 spectroscopic grade), and it was used without further purification.

**Enzymes**

HRPO (EC 1.11.1.7) was from Sigma (St. Louis, MO, U.S.A.). Two types were used: type I (80 U/mg solid) and type VII (85 U/mg solid) containing only one isoenzyme. LPO (EC 1.11.1.7) was also from Sigma (80 U/mg solid). Hydrogen peroxide (H$_2$O$_2$) was from Fluka, and ABTS was from Sigma. All these substances were kept at 4°C. Buffer solutions were made up using solutions of potassium dihydrogen phosphate (66.7 mM) and disodium hydrogen phosphate (66.7 mM), both from Prolabo (Normapur, France).

**Apparatus**

The reactions were carried out directly in spectrophotometer cuvettes (Hellma 110 QS quartz cells with 1-cm optical path length). The spectrophotometer was a double beam UV-visible instrument (Kontron 860, Uvikon, St. Quentin, France), equipped with a Peltier effect thermostated sample holder.

**Chemical experiments**

This method was performed as reported previously (3). Briefly, the solutions were made up freshly by dilution of stock solutions prepared by accurate weighing. The complexes were formed directly in the cuvettes by mixing 1.5 ml of a solution of iodine and 1.5 ml of a solution of 2T2T. Spectra were recorded immediately. The thermodynamic parameters were calculated on a microcomputer from the changes in absorbance of a series of solutions at three different temperatures 15, 20 and 30°C, using a program developed in our laboratory.

**Enzyme reaction**

The activity of HRPO and LPO was determined at 20°C and pH 7 by measuring the rate of oxidation of ABTS by hydrogen peroxide. The absorption spectrum of oxidized ABTS has a characteristic band at 411 nm.

The reaction mixture (3 ml) had the following composition: enzyme: HRPO 1.33 μg/ml or LPO 6.67 μg/ml, ABTS: $3 \times 10^{-2}$–3 mM, 2T2T: $3.3 \times 10^{-1}$ mM, H$_2$O$_2$: 1.15 mM, phosphate buffer: 66.7 mM.

Preliminary experiments established that the hydrogen peroxide concentration was saturating for the enzyme without denaturing it. The enzyme was preincubated in the test solutions for 10 min before addition of H$_2$O$_2$. The absorbance at 411 nm was recorded against a blank sample of the solution without the enzyme. The initial rate of reaction was determined from the slope of the tangent at time zero of the plot of optical density against time.

**Experiments on animals**

Male Wistar rats were supplied by Janvier (St. Berthevin, France) and were divided into groups of 10 animals for the control and 10 animals for 2T2T. Before the start of the experiments, the rats were fed a diet with a normal iodine content (10.75 μM iodine per day) with ad libitum access to water for three weeks. Then the animals received by gastric intubation a 5% suspension of the drug in gum arabic. The suspensions were prepared so that 1 ml contained 50 or 100 mg/kg, depending on the toxicity of the compound. The control group received the same volume of a 5% solution of gum arabic. After three weeks of treatment, blood samples were taken by cardiac puncture, and the levels of circulating T$_3$ and T$_4$ were determined by radioimmunoassay (RIA Gnost Behring, Behring Werke, Marburg, Germany). Then the animals were anesthetized with ether and killed. Thyroid glands were removed, sections were cut perpendicular to the larynx and fixed in Bouin's solution. Four-micron sections cut with a microtome and stained with hematein-eosin-safran.

**RESULTS**

**Chemical experiments**

Visible region: When a solution of iodine is added to a solution of 2T2T in carbon tetrachloride, the characteristic band of the halogen
is shifted to longer wavelengths (Δλ = 85 nm). This new band is referred to as a BSB. Spectra of a series of mixtures with different concentrations of 2T2T and a fixed concentration of iodine ([I₂] = 5.78 × 10⁻⁵ M) were recorded between 375 and 600 nm. The spectra crossed at an isobestic point, 491 nm (Fig. 1). The peak absorbance of the complex was determined by placing a solution of the halogen at an identical concentration to that in the complex in the reference beam. The donor 2T2T does not absorb at these wavelengths.

The formation constants Kc of the iodine 2T2T complex were evaluated from the BSB using Lang's method (5, 6). The values of the spectral and thermodynamic parameters (Table 1) were indicative of a strong molecular interaction between iodine and 2T2T (Kc = 2,527 l·mole⁻¹ at 20°C). The experimental points exactly fitted the linear regression lines on the plots of Lang's equation.

**UV region:** The UV absorption spectrum of

| T°C | λ(nm) | Ke* (l·mole⁻¹) | Mean Ke (mole⁻¹ cm⁻¹) | εc* (mole⁻¹ cm⁻¹) |
|-----|-------|---------------|-----------------------|--------------------|
| 425 | 3309  | 3084          |
| 430 | 3361  | 2995          |
| 440 | 3489  | 2634          |
| 445 | 3490  | 2408          |
| 425 | 2616  | 2939          |
| 430 | 2327  | 3042          |
| 440 | 2532  | 2659          |
| 445 | 2544  | 2414          |
| 425 | 1522  | 2850          |
| 430 | 1123  | 3448          |
| 440 | 1433  | 2667          |
| 445 | 1424  | 2450          |

ΔH° = 10.87 ± 1.44 kcal-mole⁻¹, ΔS° = 21.54 ± 5.02 cal-mole⁻¹ K⁻¹, ΔG°²98K = 4.57 ± 0.09 kcal-mole⁻¹. *Evaluated from the visible band of the complex using Lang's method with 5 solutions of complex 2T2T-I₂. [I₂] = 5.780 × 10⁻⁵ M, [2T2T] ranging from 4.638 × 10⁻⁴ M to 1.160 × 10⁻⁴ M.
2T2T in carbon tetrachloride displayed a main peak at 283 nm ($\log e = 4.18$) and a smaller peak at 256 nm ($\log e = 3.96$).

Mixing 2T2T and iodine led to the appearance of a new and more intense absorption band with a peak at 301 nm (Fig. 2). This new band is the result of the combination of the donor absorption band and that of the CTB of the complex. The CTB of the complex can be determined by placing a solution of the donor at the same concentration as that in the complex in the reference beam, and by subtracting the absorption due to iodine. It was found to have a maximum absorption at $310 \pm 0.5$ nm. The energy of the charge transfer is both a function of the ionization potential of the donor and the electron affinity of the acceptor. The ionization potential $I_p$ of 2T2T can be evaluated ($I_p = 8.75 \pm 0.01$ e.v.) using the relationships between the ionization potential and maxima frequency of the charge transfer band as described by McConnel and Hastings (7, 8).

**Enzyme reaction**

The action of 2T2T on peroxidase activity was determined by measuring the initial rate of reaction for different concentrations of substrate ABTS and 2T2T for the two enzymes HRPO and LPO. The properties of LPO are similar to those of TPO (9, 10). This commercially available peroxidase has standardized activity since TPO, not easy to isolate and purify, may be labile. 2T2T strongly inhibited both enzymes (Fig. 3). The Dixon plots were similar for the two enzymes, but were of a complex nature (Fig. 4) because some of the
curves assume a parabolic shape which could be due to the binding of several molecules of 2T2T to the enzyme. However, at low concentrations of 2T2T, the Dixon and Michaelis-Menten plots indicate competitive inhibition with an inhibition constant \( K_i \) of 5.7 mM for HRPO (Fig. 4) and 0.13 mM for LPO. A \( K_m \) of 0.4 mM was found for both enzymes with ABTS as the substrate.

**Biochemistry and histological examination**

We measured the circulating levels of T3 and T4 and examined the thyroid gland histologically after a 3-week treatment with the drug. We also measured the weights of the rats and the weights of thyroid glands. Thyroid weight was not found to be a useful indicator of antithyroid activity since even in the event of hyperactivity, the colloid content may be reduced, thereby reducing the overall weight of the gland. Furthermore, in the event of hypoactivity of the gland, the overall weight of the animal may not be affected. In practice, the appearance of the epithelium was found to be a reliable indicator of thyroid activity. Histological examination showed that hyperactivity was reflected by the presence of a cylindrical epithelium. In a normal thyroid gland, the epithelium has a cubic appearance; and an intermediate level of activity, the epithelium has a cylindrical-cubic appearance.

In our experiments, all animals had a cylindrical epithelium, indicating that the drug had strong antithyroid activity. The levels of thyroid hormones were also markedly reduced.

There was a good relationship between the value of \( K_c \) and the histological appearance (Table 2).

**DISCUSSION**

2T2T is a synthetic antithyroid agent with moderate electron donor properties \( (K_c = 25271 \text{ mole}^{-1}) \). The alkaline thiocyanates which have weak antithyroid activity have a \( K_c \) of around 96 \text{ mole}^{-1}, while MMI, the

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**Fig. 4.** Dixon plots of 2-thiazoline-2-thiol (2T2T) inhibition of the reaction catalyzed by horseradish peroxidase (1.33 \( \mu \text{g/ml} \)) and different concentrations of 2-2' azino-bis-(3)-ethylbenzothiazolinesulfonic acid: ▲ 1.39 \( \times 10^{-4} \text{ M} \), ○ 8.231 \( \times 10^{-5} \text{ M} \), ■ 5.54 \( \times 10^{-5} \text{ M} \), ● 4.16 \( \times 10^{-5} \text{ M} \), □ 2.77 \( \times 10^{-5} \text{ M} \). Conditions are described in the text. A similar curve was obtained with lactoperoxidase.
strongest antithyroid agent known, has a $K_c$ of 23,194 l-mole$^{-1}$ (11). The electron donor properties confer an action on molecular iodine $I_2$. It is thought that significant amounts of molecular iodine can be formed in the absence of receptor during oxidation of iodide (12, 13). It has also been shown that the components required for iodination $I^-/H_2O_2/TPO$ are not in the same compartment as thyroglobulin (14). Thus $2T2T$ could form a charge-transfer complex of the $n$-$\sigma$ type and 1:1 stoichiometry with molecular iodine in the thyroid gland. This would effectively prevent or inhibit oxidation to $I^+$, thereby inhibiting hormone synthesis. Furthermore, the $2T2T$-$I_2$ complex (outer complex) is slowly transformed into a stable inner complex liberating $I^-$ ions.

$$2T2T + I_2 \rightarrow 2T2T-I_2 \rightarrow 2T2T-I^+ - I^-$$

These $I^-$ ions will tend to react with free iodine in the thyroid gland to produce $I_3^-$ ions that are unable to bind to tyrosine residues. The electron donor activity also enables the drug to act on TPO. It has been shown that MMI can bind covalently to the heme iron of TPO (15) by transfer of electrons to the iron atom. We found that $2T2T$ also inhibited lactoperoxidase.

It is thus likely that $2T2T$ also inhibits TPO. Inactivation of this enzyme will block oxidation of circulating $I^-$ to $I_2$ and to the $I^+$ required for iodination of the tyrosine residues of thyroglobulin. This action would also inhibit hormone synthesis. For $2T2T$, the two actions appear to take place (Fig. 5) since the moderate ability to complex iodine and the action on peroxidase concurred to produce a dramatic effect in vivo with a marked reduc-

![Fig. 5.](image-url)
tion in thyroid hormone levels and significant hyperactivity of the gland.

The in vitro results either on complexation of iodine or inactivation of peroxidase would not have suggested such a strong action in vivo. Structure-activity relationships could thus be established with respect to electron donor power, which could aid development of new antithyroid agents. Conversely, a number of drugs have unwanted antithyroid activity. Compounds with a structure that hinders attack by iodine may thus be expected to have antiperoxidase activity. On the other hand, strong nucleophiles would be more likely to complex molecular iodine.

The example represented by 2T2T indicates the possibility of a synergistic action between its activity towards iodine and the action on TPO, which represents a new interpretation of the mechanism of action of this drug and those of antithyroid agents in general. This combined mode of action needs to be taken into account in any evaluation of the activity of new antithyroid agents.

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