Anionic iodotyrosine residues are required for iodothyronine synthesis

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Abstract

Biosynthesis of iodothyronines in thyroglobulin occurs by oxidative coupling of two iodotyrosine residues catalyzed by thyroperoxidase. To study the mechanism of iodothyronine formation, iodine-free thyroglobulin was non-enzymatically iodinated and after removal of non-incorporated iodide, incubated with lactoperoxidase and glucose oxidase between pH 4 and 9. The amount of thyroxine (T_4), 3,5,3′-tri-iodothyronine (T_3), 3,3′,5′-tri-iodothyronine (rT_3) and 3,3′-di-iodothyronine (T_2) formed was measured by radioimmunoassays after hydrolysis of thyroglobulin.

T_4 is synthesized out of two di-iodotyrosine (DIT) residues in thyroglobulin. The pH dependence of T_4 formation fits the dissociation curve of the DIT phenoxy group (pK_a 6.5).

The formation of T_2, synthesized out of two mono-iodotyrosine (MIT) residues, shows a quite different pH dependence. Below pH 6, T_2 synthesis could not be observed, while above pH 7.4 a relatively large increase occurred. The values up to pH 8 fitted the dissociation curve of the MIT-phenoxy group with a pK_a of 8.7. The gradual loss in enzymatic activity of peroxidase and oxidase in the reaction made the values obtained above pH 8 unreliable. The importance of the ionization of the phenoxy group for the coupling reaction was further consolidated by showing that the pH-dependent oxidation of 2-methoxy-phenol (guaiacol) had 50% maximal product formation at pH 7, a value concordant with pK_a 7.0 for the ionization of the phenoxy group of this agent.

T_3 and rT_3 synthesis followed mainly the ionization curve of the inner-ring hydroxyl group, indicating that this ring has the greatest influence on hormonogenesis. Since anion formation facilitates the removal of an electron under oxidative conditions, the pH dependence agrees with the involvement of phenoxy radicals in iodothyronine synthesis, a process that most likely also occurs *in vivo* since it is mainly T_4 that is formed in thyroglobulin.

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Introduction

Thyroglobulin (Tg), a thyroidal glycoprotein, is the protein dimer in which iodothyronines are synthesized (1). Thyroxine (T_4) is the main product, but some 3,5,3′-tri-iodothyronine (T_3) and even low amounts of 3,3′,5′-tri-iodothyronine (rT_3) are also formed. Synthesis of iodothyronines occurs by coupling of iodinated tyrosine residues (2). T_4 is formed by coupling of two di-iodotyrosine (DIT) residues, while coupling of mono-iodotyrosine (MIT) with DIT gives T_3 or rT_3. The outer ring in T_3 is mono-iodophenol, in rT_3 it is di-iodophenol. In principle, other iodothyronines can also be formed such as 3,3′-di-iodothyronine (T_2) by coupling of two MIT residues. Both the iodination and the coupling reactions are catalyzed by thyroperoxidase (TPO). *In vitro*, TPO can be replaced by other peroxidases such as lactoperoxidase (LPO) (3–5). Iodination is thought to take place by a two-electron-transfer reaction in which

an enzyme–OI^- complex (5) or free OI^- (6) is the reactive intermediate. There are strong arguments that, in thyroid hormonogenesis, phenoxy radicals are involved (2, 7, 8), but an ionic mechanism has also been described (9). Both the iodination and the coupling reactions are pH dependent (10–12). In this paper the coupling reaction dependent on pH is described and we conclude that, for thyroid hormonogenesis, ionization of phenylhydroxyl groups is required.

Materials and methods

Thyroglobulin preparation

To obtain iodine-free Tg (13), Tg was purified from a human thyroid gland obtained after surgery from a patient with a total iodide organification defect (TIOD). Informed consent to use this material for scientific research was obtained.

For purification of Tg, about 1 g thyroid tissue was pulverized under liquid N_2 and homogenized in phosphate-buffered saline (PBS: 9.2 mmol/l Na_2HPO_4, 1.3 mmol/l NaH_2PO_4, 140 mmol/l NaCl, pH 7.4) containing phenyl-methyl sulfonyl fluoride (10 mg/l) and 1% aprotinine. After centrifugation at 100 000 *g* the supernatant was brought to 50% ammonium sulfate saturation, incubated at 4 °C for 16 h and centrifuged at 10 000 *g*. The precipitated Tg was dissolved in and dialyzed against PBS followed by chromatography on Sepharose CL-6B. Fractions containing Tg were pooled and concentrated with an Amicon YM100 filter (Amicon, Danvers, MA, USA).

Protein concentration of the Tg preparation was measured using bicinchoninic acid (Pierce, Rockford, IL, USA) as reagent (14). Iodine content of Tg was measured according to de Vijlder *et al*. (15). Briefly, Tg was denatured with $HClO_3$ and the iodine content was determined spectroscopically with the cerium-arsenite method by measuring the slope of the log extinction at 420 nm as a function of time.

Iodination

To obtain iodinated Tg with a low content of iodothyronines, the iodine-free human Tg, obtained from the patient with a TIOD, was non-enzymatically iodinated with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen) (Pierce, Rockford, IL, USA). Tubes were coated with Iodogen according to the manufacturer's manual, under nitrogen flow. For iodination the reaction mixture in the Iodogen-coated tubes contained 2 μg iodine-free Tg/μl, 0.1 mmol/l KI and 1 mmol/l NaH_2PO_4/ Na_2HPO_4, pH 7.0, together with a tracer dose of $^{123}I^-$ or $^{125}I^-$. After incubation at room temperature for 8 min, non-incorporated iodide was removed by ultradialyzing the sample five times on a YM30 Amicon filter (centricon tubes, Amicon). Iodine incorporation in Tg was determined in a sample after protein precipitation with trichloroacetic acid.

Coupling of iodotyrosine residues

After iodination of the Tg with Iodogen the pH was changed by adding 50 mmol/l NaH_2PO_4/Na_2HPO_4 in the pH range 4–9. The iodothyronine synthesis in Tg (1.25 μg/μl) was started by adding a mixture of LPO (0.1 U/ml; Sigma Chemical Co., St Louis, MO, USA), glucose oxidase (0.1 U/ml; Sigma) and 10 mmol/l glucose (final concentrations). After incubation at 37 °C for 30 min the reaction was stopped by adding 0.25 mol/l methimazol. The samples were hydrolyzed with pronase E (Serva, Heidelberg, Germany) and leucine aminopeptidase (Sigma) (16). To check the hydrolysis and to measure the amount of DIT and MIT in the samples thin layer chromatography (TLC) was performed (17, 18). Briefly, an enclosed glass chamber was equilibrated with tertiary amylalcohol:tertiary butanol:12 mol/L ammonia:acetone (10:20:20:80). Of each sample iodinated with ^{125}I, 10 μl was applied to the TLC plates (No. 5582 from Merck, Darmstadt, Germany) together with markers for MIT, DIT and other iodothyronines. The plates were run for 2 h in the chamber. After chromatography the plates were dried. TLC plates were autoradiographed by a phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA), which was scanned by a Molecular Dynamics Phosphor Imager. Data were analyzed by ImageQuant software (Molecular Dynamics). T_2, rT_3, T_3 and T_4 concentrations in the samples were measured quantitatively by radioimmunoassays (RIAs) (19–21), and related to the protein concentrations.

Results

Tg obtained from the patient with TIOD did not contain detectable iodine. After iodination of Tg with Iodogen the amount of iodine incorporated was about 20 atoms of iodine per molecule Tg in 8 min. The iodine was divided over about 6 mol MIT/mol Tg, 5 mol DIT/mol Tg and 0.5 mol iodothyronines/mol Tg. The non-enzymatic iodination of Tg, after removal of non-incorporated iodide, was followed by LPO-catalyzed iodothyronine synthesis at various pH values. T_4 formation markedly increased between pH 4 and 8, with a half-maximal value at about pH 6.3 (Fig. 1). At pH 7.2 an increase of 0.5 mol T_4/mol Tg was observed. T_2 was formed at pH values higher than 6. At pH 7.2 only 0.01–0.02 mol T_2/mol Tg could be measured. At higher pH, especially above pH 7.4, a rise in T_2 synthesis could be observed. T_3 and rT_3 formation also occurred in the pH range 4–8. For rT_3 synthesis a relatively strong increase was observed above pH 7.4, while T_3 showed a maximum at about pH 8, with a half-maximal value at about pH 6.5. The increase in T_3 and rT_3 at pH 7.2 was 0.08 and 0.14 mol/mol Tg respectively. As can be seen in Table 1, both LPO and H_2O_2 were required for iodothyronine synthesis; in the absence of peroxidase some minor iodothyronine formation might still occur, but LPO without H_2O_2 did not result in any iodothyronine formation.

Discussion

In this paper we show that the coupling of iodotyrosine residues to iodothyronines is pH dependent. The pH dependence of T_4 formation follows a curve reaching a maximal value at about pH 8 with the half-maximal value at about pH 6.3 near the dissociation curve of the DIT phenoxy group (pK_a). The observed T_4 values up to pH 8 did indeed follow the pH-dependent ionization of the hydroxyl group of DIT ($pK_a \approx 6.5$) (22) (for structural formulae see Fig. 2). T_2 synthesized out of two MIT residues also shows pH dependence. Below pH 6 no synthesis could be seen and a relatively strong increase was observed above pH 7.4. The curve up to pH
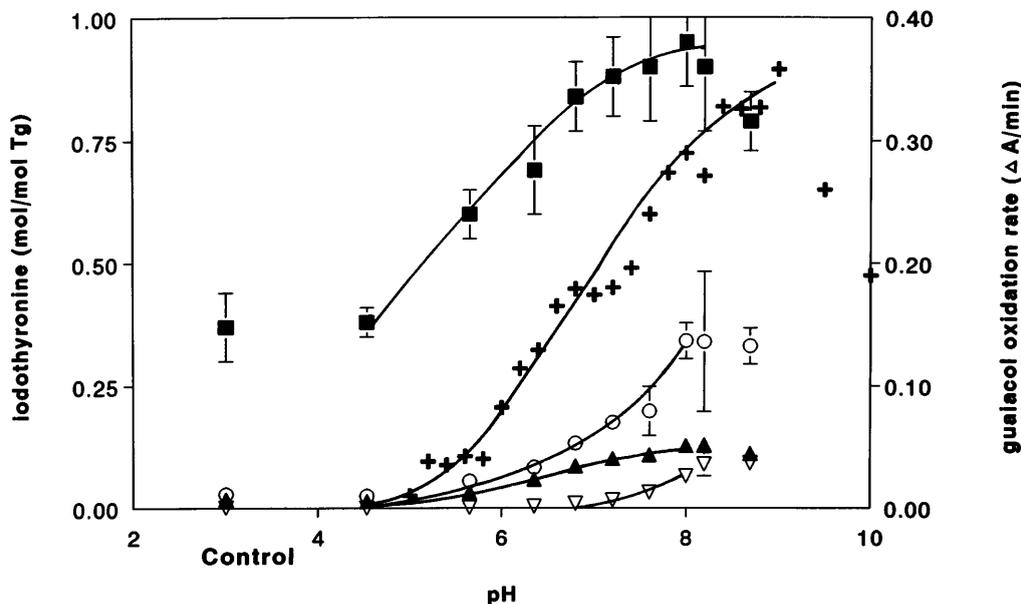
Figure 1 Iodothyronine formation. Iodogen-iodinated Tg was incubated with LPO and glucose oxidase at various pHs (for conditions see Materials and methods). Control incubation was carried out at pH 7.2 without LPO or glucose oxidase. T_4 (■), T_3 (▲), rT_3 (○) and T_2 (▽) formation in four independent incubations ($n=4\pm$S.D.) were measured by RIAs after proteolysis of Tg. The guaiacol oxidation rates (+) are plotted on the right ordinate (ΔA/min) against the pH.

8 fits the dissociation curve of the MIT phenoxy group ($pK_a \approx 8.7$) (22). Above pH 8, T_2 synthesis could not be measured correctly because of a loss in peroxidase and oxidase activity (23) and possibly because of changes in the structure of Tg (24).

To investigate whether the phenoxy group is really involved in the coupling reaction, we used the 2-methoxy-phenol (guaiacol) oxidation reaction. We plotted the guaiacol oxidation rates as given by Kootstra *et al.* (12) against pH. The curve of product formation gave a half-maximal value at pH 7.0 (Fig. 1), a value equal to the pK_a 7.0 of the phenoxy group of guaiacol (25).

The pH dependence of T_3 and rT_3 formation is more complex. T_3 synthesis follows a curve with a maximum at pH 8 and a half-maximal value at about pH 6.5, indicating that in this reaction the inner ring, being the DIT residue, acts as a free radical with the C1 of the MIT residue under formation of a radical complex that subsequently is oxidized to T_3 (Fig. 3). In contrast to T_3 formation, the formation of rT_3 increased more strongly

above about pH 7.4. Above pH 8 denaturation starts to occur and no further increase could be observed. However, considering the form of the curve in Fig. 1, it is unlikely that at pH 8 the maximum synthesis has been reached. Apparently, in this case too the ionization of the inner ring (MIT) phenoxy group has most influence on the synthesis rate. After ionization and radical formation MIT reacts with the DIT radical to an intermediate complex, which converts to rT_3 (Fig. 3).

In contrast to T_2 formation, rT_3 formation could already be observed at pH 6. This may be possible if ionization of the DIT residue and consequent radical formation has an influence on rT_3 formation.

Our results agree with findings *in vivo,* where Tg (containing $6-33$ atoms iodine/molecule Tg) isolated from thyroid glands contains mainly T_4 (1.0–4.5 mol/mol), less T_3 (0.05–0.37 mol/mol) and rT_3 (0.02–0.11 mol/mol) and only trace amounts of T_2 (0.02 mol/mol) in highly iodinated Tg (26). These findings suggest that the iodotyrosine residues in Tg involved in the coupling reaction are located at the outside of the

Table 1 Effect of lactoperoxidase (LPO) and glucose oxidase (GOD) on iodothyronine formation incubated at pH 7.2. The iodothyronines were measured by radioimmunoassays. Values are means$\pm$ s.D. ($n=4$).

| Conditions	T_4 (mol/mol Tg)	T_3 (mol/mol Tg)	rT_3 (mol/mol Tg)	T_2 (mol/mol Tg)
$-$LPO $+$ GOD	0.4 ± 0.0	18.7 ± 2.0	35.4 ± 3.1	1.4 ± 0.2
$-$LPO $-$ GOD	0.4 ± 0.1	17.7 ± 4.2	28.1 ± 5.3	0.9 ± 0.2
$+$LPO $+$ GOD	0.9 ± 0.1	101.0 ± 13.0	174.9 ± 7.0	16.2 ± 0.9
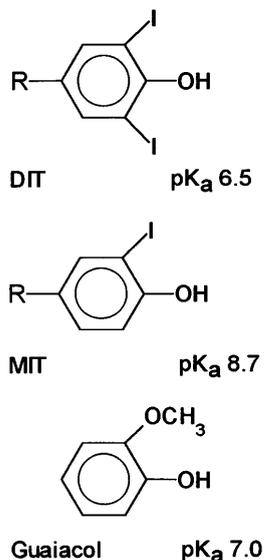
Figure 2 Structural formulae of DIT, MIT and guaiacol, together with the pK_a values.

molecule and that *in vivo* the coupling reaction takes place at a pH near the physiological value. In that case most DIT residues will be ionized ($\approx$ 90%) while only about 5% of MIT residues will be ionized, explaining why it is mainly T_4 production which takes place.

Summarizing, the coupling of iodotyrosine residues requires anion formation which is pH dependent. The coupling of iodotyrosine residues can be explained by a free radical mechanism (2, 7, 8) or by an ionic mechanism (9). Although our experiments cannot make a distinction between these mechanisms, our results fit the concept that thyroid hormonogenesis requires phenoxy anions promoting radical formation. Since DIT is largely ionized at physiological pH, it might explain why it is mainly T_4 which is formed under physiological conditions.

Acknowledgements
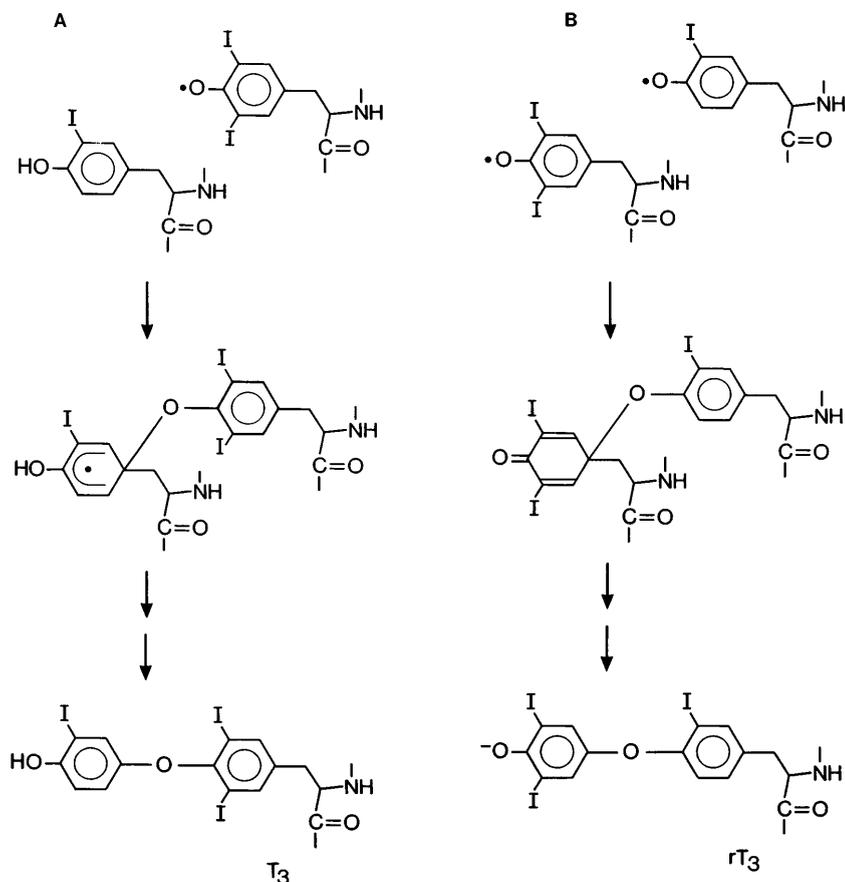
Figure 3 Schematic representation of (A) T_3 and (B) rT_3 formation.

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