Tyrosine 130 Is an Important Outer Ring Donor for Thyroxine Formation in Thyroglobulin*

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The thyroid couples two iodotyrosine molecules to produce thyroid hormone at the acceptor site in thyroglobulin, leaving dehydroalanine or pyruvate at the donor position. Previous work has located the acceptors but not the principal iodotyrosine donors. We incorporated [14C]tyrosine into beef thyroid slices, isolated and iodinated the [14C]thyroglobulin (Tg I), separated its N-terminal ~22-kDa hormone-rich peptide, and digested the latter with trypsin and endoproteinase Glu-C (EC 3.4.21.19). Nonlabeled thyroglobulin (Tg II) was isolated from the same glands and processed similarly, without iodination in vitro. Tg I was used to initially recognize pyruvate in peptide fractions, and Tg II was used to then identify its location in the thyroglobulin polypeptide chain. Sequencing of a tryptic peptide by mass spectrometry and Edman degradation showed a cleavage after Val129. An endoproteinase Glu-C-generated peptide had the predicted molecular mass of a fragment containing residues 130–146 with Tyr130 replaced by pyruvate; the identification of this peptide was supported by obtaining the expected shortened fragment after tryptic digestion. [14C]-labeled pyruvate was identified in the same fraction as this peptide. We conclude that Tyr130 is an important donor of the outer iodothyronine ring. Its likely acceptor is Tyr5, the most important hormonogenic site of thyroglobulin, because Tyr5 and Tyr130 are proximate, because they are the most prominent early iodination sites in this part of thyroglobulin, and because the N-terminal region was previously found capable of forming T4 by itself.

Thyroid hormone synthesis involves a two step modification of tyrosyl residues within the 2750-residue polypeptide chain of bovine Tg1 (1). First, certain tyrosyls are iodinated to form the hormone precursors MIT and DIT, and then two iodinated tyrosyls couple to form the biologically active iodothyronines T3 and T4. In this latter reaction, a tyrosyl donates its iodinated phenyl group to become the outer ring of the iodothyronine residue at an acceptor site, leaving DHA or its derivative at the donor position (2, 3). Both iodination and subsequent coupling are mediated by a thyroperoxidase at the apical membrane of the cell (1).

The most important T4 forming site in all vertebrate species examined is at Tyr6. It appears in a 20–26-kDa N-terminal peptide released by reduction of mature Tg (4–9). Two additional hormonogenic sites occur in the C-terminal region at Tyr3264 and Tyr3274 (human numbering) (7–11) as well as at a mid-chain site at Tyr1290 (7, 8, 12). Priority for hormonogenesis at these sites varies among species (13) and under different physiological conditions, including iodine availability and TSH stimulation (8, 9). Far less is known about the tyrosyls that contribute the outer iodoaryl ring of the iodothyronines. Gavaret et al. (3), using [14C]-labeled Tg from pig thyroid slices, concluded that the residual side chain of the donor existed as DHA while in peptide linkage and was converted to Pyr after its release from Tg by enzymatic digestion, or to acetate after acid hydrolysis. Locating the origins of these donors within Tg has been difficult. Proposed sites, all resting on indirect evidence, have included the Tyr2469 or Tyr2522 of bTg, based on the conversion of DHA either to labeled Ala by borohydride reduction or to labeled Asp by Na14CN (14), and Tyr residues 5, 926, 986, 1008, and 1375 of bTg, from 4-aminothiophenyl modification of DHA residues (15). Marriq et al. (16) iodinated in vitro a CNBr-derived peptide from low iodine hTg and concluded that Tyr130 was converted to DHA with donation of an iodoaryl ring for iodothyronine formation at Tyr6. However, Xiao et al. (17), using the same substrate under similar experimental conditions, found that Tyr130 served as acceptor rather than donor, suggesting that the CNBr peptide is not a good model for hormonogenesis in intact Tg. Using a different approach based on graded iodination in vitro of low iodine hTg, we identified three potential donors, at Tyr5, and used within 1–3 h of death. We made two Tg preparations: Tg I, from thyroid slices incubated with [14C]Tyr and subsequently iodinated in vitro; and Tg II, isolated directly from the same beef thyroid without

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1 The abbreviations used are: Tg, thyroglobulin; hTg, human and bovine Tg, respectively; T4, thyroxine; T3, 3,3'-triiodothyronine; DIT, 3,5-diiodotyrosine; MIT, 3-iodotyrosine; Pyr, pyruvate; DHA, dehydroalanine; endo-Glu-C, endoproteinase Glu-C; CAD/MS, collisionally activated dissociation mass spectrometry; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

2 The abbreviations used are: Tg, thyroglobulin; hTg, human and bovine Tg, respectively; T4, thyroxine; T3, 3,3'-triiodothyronine; DIT, 3,5-diiodotyrosine; MIT, 3-iodotyrosine; Pyr, pyruvate; DHA, dehydroalanine; endo-Glu-C, endoproteinase Glu-C; CAD/MS, collisionally activated dissociation mass spectrometry; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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[14C]Tyr incorporation and without subsequent iodination. For Tg I, 2 g of thyroid slices were incubated in 10 ml of a tyrosine-free medium (Life Technologies, Inc., Selectamine) containing 500 μCi of uniformly labeled [14C]Tyr (500 μCi/μmol, NEN Life Science Products) at 37 °C in a water-saturated atmosphere of air-CO2 (95:5) for 20 h, then rinsed and briefly homogenized in 0.05 M sodium phosphate buffer, pH 7.4, followed by centrifugation at 105,000 × g for 1 h. Thyroglobulin was isolated from the resultant supernatant fraction at 4 °C on a Sephacryl S300 column (2.5 × 100 cm) equilibrated in the same buffer. To obtain Tg II, we processed 5 g of thyroid tissue in the same manner without prior incubation. The protein content of isolated Tg was determined using bovine serum albumin as standard (18) and its iodine content by a modification of the Sandell-Kolthoff method (19).

Iodination of Tg I—Iodination was carried out by exposure of Tg to iodine at a ratio of 20 atoms of iodine/molecule of 660-kDa Tg. We incubated 3.5 mg (5.3 nmol) of [14C]-labeled Tg in 4 ml of 0.025 M sodium phosphate buffer, pH 7.0, containing: 1.6 mg of glucose (Calbiochem); 15 μg of glucose oxidase, 296 units/mg (Calbiochem); 17.6 μg (106 nmol) of KI; and 162 μg of lactoperoxidase (Sigma). The reaction was started by the addition of glucose oxidase. Carrier-free 125I (0.05 μCi) was added to an aliquot (50 μl) of this mixture at the beginning of the incubation. Iodination, with and without added 125I, was carried out at 37 °C for 1 h and the reaction stopped by the addition of 0.02% sodium azide. The 125I-labeled sample was used directly to determine protein bound 125I and 125I iodoamino acid distribution after Pronase digestion by paper chromatography (4). Excess reagents and unreacted iodine in the non-125I-labeled sample were removed by chromatography on a column (1.5 × 100 cm) of Sephacryl S300 in sodium phosphate buffer, pH 7.4, containing 0.02% sodium azide. The peptide composition of 14C-labeled Tg (Tg I) following iodination and of nonlabeled Tg (Tg II) was analyzed by SDS-PAGE on 4–20% gradient gels under reducing conditions (20). Gels were stained with Coomassie Brilliant Blue and 14C-labeled gels

![Figure 1](image1.png)

**FIG. 1.** Separation of Tgs I and II on Sephacryl S300. **A**, Tg I, thyroglobulin-containing fractions were identified by SDS-PAGE and pooled as indicated. The late peak (tube 110) corresponds to free [14C]Tyr. Inset, autoradiogram of isolated Tg I after iodination. Molecular mass markers are indicated in kDa. **B**, Tg II; inset, SDS-PAGE of isolated Tg fraction, Coomassie Blue stained.

![Figure 2](image2.png)

**FIG. 2.** Distribution of 14C-labeled compounds following Pronase digestion from Tg I before (A) and after (B) iodination. Separation was by reverse phase-HPLC using a trifluoroacetic acid/acetonitrile gradient.

### Table I

| Fraction | Pyr | Tyr | Unk | MIT | DIT | TH |
|----------|-----|-----|-----|-----|-----|----|
| Tg(non-iodinated) | 0.1 | 91.2 | 4.6 | 1.1 | 0.2 | 0.1 |
| Tg(iodinated)* | 0.7 | 69.2 | 4.2 | 11.7 | 5.0 | 2.6 |
| FI (67%)** | 0.5 | 68.2 | 4.8 | 10.5 | 5.9 | 1.7 |
| FII (22%)** | 0.6 | 58.9 | 6.1 | 12.6 | 6.6 | 2.1 |
| FIII (8.1%)** | 2.7 | 66.4 | 3.2 | 7.8 | 3.6 | 8.0 |
| FIV (2.5%)** | 2.3 | 59.1 | 3.4 | 5.3 | 3.4 | 14.6 |

*14C-labeled Tg I was iodinated at 20 atoms of iodine/molecule of Tg as described under “Experimental Procedures.”

**Percentage in parentheses represents the fraction of total 14C recovered from the S200 column.
subjected to autoradiography after treatment with EN3HANCE™ (NEL Life Science Products). Molecular mass markers were myosin (200 kDa), galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa).

Selective Proteolysis of the N-terminal 22-kDa Peptide of Tg and Isolation of the Products—The 22-kDa peptide fractions from Tg I and from Tg II were each digested with 1:1-tosylamide 2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at an enzyme to substrate ratio of 1:50 (w/w) in 0.1 M NH₄HCO₃, 0.1 mM CaCl₂ for 1 h at 37 °C, and the reaction stopped with soybean trypsin inhibitor (Sigma). Tryptic peptides were first fractionated on a column (1.5 x 60 cm) of Bio-Gel P-6 (Bio-Rad), and selected fractions were further separated by reverse-phase HPLC using a Sephasil C8 column (Amersham Pharmacia Biotec) and a gradient of 0–90% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Standards used were Tyr, MIT, DIT, T₃, T₄, and a 14C-labeled Pyr (16 Ci/mmol, NEN Life Science Products). The fraction corresponding to Pyr was then eluted isocratically on an ion exclusion column (Aminex HPX-87H, Bio-Rad) with 0.0025 N H₂SO₄ at a flow rate of 0.6 ml/min. Oxalic, citric, malic, succinic, formic, and acetic acids (Bio-Rad) were used as standards in addition to Pyr and ¹⁴C-labeled Pyr. Detection was by ¹⁴C-label content or A₂₁₀. Pyruvate eluted with a retention time of 7.70 min, between citrate (7.50 min) and malate (9.04 min).

Analysis of Peptides by Edman Degradation and Mass Spectrometry—Tg I was used to identify ¹⁴C-Pyr-containing peptides. The same fractions isolated in parallel from Tg II were then used for further analysis by mass spectrometry. The molecular masses of selected peptides were determined in a Finnigan-MAT TSQ7000 system with an ion spray—tandem mass spectrometry with argon as the collision gas and in some cases also by mass spectrometry using electron spray—tandem mass spectrometry with argon as the collision gas and in some cases by automated Edman degradation on an Applied Biosystems 470A Protein Sequencer as described previously (7).

RESULTS

We obtained 9 mg of Tg I (1.2 million dpm/mg of protein) from thyroid slices incubated with ¹⁴C-Tyr. Twenty-three percent of ¹⁴C in the soluble fraction of labeled slices was recovered in Tg I (Fig. 1A). The yield of Tg II was 130 mg (Fig. 1B).

We iodinated 8.1 mg of Tg I using 20 atoms of iodine/mole- cule 660-kDa Tg. The insets in Fig. 1 show the pattern on gel of Tg I (Fig. 1A) after iodination and of Tg II (1B). A small aliquot of Tg I, iodinated in the same way but with added carrier-free NaI⁻¹³¹, showed 91% incorporation of ¹²⁵I into protein, including 14% as T₄ and 8% as T₃. Tg I contained 7.9 ng of iodine/µg of protein in vivo as determined by ¹²³I radioactivity after iodination and 9.2 ng of ¹²⁵I/µg of protein after iodination, compared with 9.8 ng of ¹²⁵I/µg of protein in Tg I. We identified Tg I by SDS-PAGE of ¹⁴C-labeled fractions FI-FIV (see below) and confirmed the identity of the compound in this peak by showing in a subsequent separation step by ion exclusion chromatography that it co-eluted with authentic Pyr.

Tg I and Tg II were individually reduced and alkylated, and then each was separated into four fractions (FI-FIV) by size exclusion chromatography (shown in Fig. 3 for Tg I). Fractions I and II contained primarily peptides of >200 kDa (Fig. 3, inset) and several intermediate bands of 200–40 kDa. Fraction III contained a major band of ~22 kDa and several smaller bands. Our designation of ~22 kDa is based on its migration on gel; it appears the same as the 30 kDa described by Gregg et al. (22), who perhaps calculated that mass from its constituents. We identified the ~22 kDa as an N-terminal peptide normally released from Tg by reduction, on the basis of its size, its high hormone content (Table I), and from sequencing some of its tryptic peptides (see below). Fraction IV contained a major band of ~10 kDa, together with bands of ~22 and 20 kDa. Its high TH content suggested this fraction also contained N-
terminal peptides of Tg. Both fractions III and IV had a high Pyr content after Pronase digestion (Table I).

Fractions III from both Tg I and Tg II were selected for further study, separately but in parallel. Each was digested with trypsin, and the resultant peptides were separated into three fractions on a size-exclusion column (Fig. 4 for Tg I). Of these, fraction IIIc (from Tg I) had the highest [14C]Pyr content (2%, compared with 1% in fractions IIIa and IIIb), and on HPLC it resolved into at least 20 peptides (Fig. 5A) and 8 [14C]-labeled peaks (Fig. 5B, A–H). Fractions corresponding to each of the [14C]-labeled peaks were analyzed by one or more methods, including the distribution of [14C] in Pronase-digested material (from Tg I) and by Edman degradation (from Tg I) or by mass spectrometry (from Tg II) (Table II), and were related to the theoretical tryptic peptides of N-terminal bTg, shown in Fig. 6. The high [14C]Pyr content of Peak B suggested that it included a donor-containing peptide. It was well separated from a free Pyr standard in this HPLC system. Edman degradation identified several peptides in this peak but none had Tyr in the cDNA-derived sequence of bTg, suggesting that the putative donor peptide may have been blocked at its N terminus and therefore unreadable. Peptide T8 (Fig. 6) was

![Figure 5](image)

**Figure 5.** Separation of tryptic peptides of FIIIc from Tg I (Fig. 3) on HPLC using a C8 column and an ammonium bicarbonate-acetonitrile gradient. Flow rate was 1 ml/min with 1-min collections. A, peptide distribution, A<sub>214</sub>; B, [14C] distribution.

**Table II**

| Peak | Percent of total [14C] | Tryptic peptide<sup>a</sup> | Identification method<sup>b</sup> |
|------|------------------------|-----------------------------|----------------------------------|
| A    | 4%                     | 91%                         | ND<sup>c</sup>                   |
| B    | 28%                    | 69%                         | T8(fragment), T10, T11           | Edman (Tg I) |
| C    | —                      | 100%                        | ND                               |
| D<sup>d</sup> | 1%                   | 44%                         | 6%                               | ND            |
| E    | —                      | 97%                         | ND                               |
| F    | 2%                     | 66%                         | T8(fragment residues 118–129)    | Edman (Tg I), CAD/MS (Tg II) |
| G    | —                      | 80%                         | T17, T18                         | CAD/MS (Tg II) |
| H    | —                      | 13%                         | T1                               | [14C] distribution (Tg I) |

<sup>a</sup> Theoretical tryptic peptides as shown in Fig. 6.

<sup>b</sup> Fractions corresponding to [14C] peaks A–H were analyzed by one or more methods including [14C] distribution after Pronase digestion and sequencing by Edman degradation or by CAD/MS. The Tg preparation used is indicated in parentheses.

<sup>c</sup> Not determined.

<sup>d</sup> Peak D contained 24% of its [14C] as MIT + DIT.
identified in truncated form ending immediately before residue 130 (Tyr) by Edman degradation of material from Tg I and by mass spectrometry of this peptide from Tg II. The measured molecular mass of the latter was 1459.6 ± 0.5 Da, identical to the theoretical mass of residues 118–129 with the N-terminal Gln residue converted to a pyrolline carboxylic acid, an expected consequence of the acidic conditions used during the analysis. The CAD spectrum for this peptide from Tg II confirmed its sequence and the absence of residues after Val129.

Samples of fraction IIIc (Fig. 4) from both Tg I and Tg II were also digested with endo-Glu-C, and the resultant peptides were separated on HPLC into 18 or more fractions (Fig. 7A for Tg II) and 5 14C-labeled peaks (Fig. 7B, for Tg I). The distribution of radioactivity after Pronase digestion of each peak from Tg I is summarized in Table III. Peak C2 had a high [14C]Pyr content. On capillary HPLC, peak C2 from Tg II separated into five peptide peaks (Fig. 8A), and the molecular masses of peaks 1–4 in Fig. 8A were determined by mass spectrometry. The measured molecular mass of peptide 1 was 1981.6 ± 1.3 daltons, which corresponds closely to the mass of a modified endo-Glu-C peptide (1980.02 daltons) of bTg (Fig. 9A) with the expected loss of Val129 from the cleavage described above and the substitution of Pyr or DHA at Tyr130 (Fig. 9B). Pyr is more likely than DHA because the mass spectrum shows a fragmented form of the peptide (Fig. 8B) corresponding to a loss of 43 daltons, consistent with removal of the acetyl group of Pyr.

| Peak | Pyr | Tyr | Unk | MIT | DIT | TH |
|------|-----|-----|-----|-----|-----|----|
| A    | 3   | 62  | 4   | 8   | 6   | 3  |
| B    | 2   | 73  | 4   | 7   | 5   | 2  |
| C2   | 9   | 78  | 0   | 2   | 3   | 0  |
| C3   | 13  | 64  | 0   | 2   | 3   | 3  |
| D    | 3   | 38  | 6   | 7   | 4   | 28 |
| E    | 3   | 44  | 11  | 15  | 5   | 5  |
of the modified endo-Glu-C peptide, an expected product of trypsin digestion (Fig. 9C) with a theoretical mass of 1607.7 daltons. The mass spectrometry for this peptide also showed it in fragmented form with a 43 dalton loss. Peak 3 (Fig. 8A) was identified by molecular mass and CAD/MS, as Tg residues 212–225, an expected product of endo-Glu-C digestion. Peaks 2 and 4 did not contain identifiable peptides and peak 5 was not analyzed.

**DISCUSSION**

We report a new approach to the problem of locating a few iodothyronine outer ring donor sites within the 2750-residue polypeptide chain of Tg. First we isolated peptide fractions containing [14C]Pyr after in vitro iodination of Tg labeled with [14C]Tyr. This led to the identification of Pyr-containing peptides from naturally iodinated Tg isolated in parallel to the 14C-labeled Tg, using mass spectrometry and referring to the cDNA-derived primary structure (23). The Pyr is presumably a derivative of DHA (2, 3). Our results identify the Tyr130 of bTg as donor of an outer iodothyronine ring. We base this conclusion on the following: (a) a truncated trypsin peptide ending immediately before residue 130, as identified in Tg I by Edman degradation and in Tg II by CAD/MS, suggested an unstable modification of this residue; (b) an endo-Glu-C peptide from Tg II had the predicted molecular mass of a peptide containing residues 130–146, with Tyr130 replaced by Pyr; (c) trypsin digestion shortened this peptide to a size predicted from the loss of a cleaved tripeptide consisting of residues 144–146; and (d) the same HPLC fraction (Fig. 7B, peak C2) from endo-Glu-C digested FIII of Tg I contained [14C]Pyr. This direct identification of Tyr130 as donor agrees with predictions made from indirect evidence by Marriq et al. (16) and by us (9). Support for the physiological importance of this donor site comes from a goitrous hypothyroid family described by Ieiri et al. (24); its members lacked exon 4 of Tg, which codes for a 70-residue segment including Tyr130, suggesting that Tyr130 was necessary for adequate production of thyroid hormone.

Mass spectrometry and Edman degradation sequencing of trypsin peptide T8 of bTg (Fig. 6) showed the expected sequence only through residue 129, suggesting peptide cleavage between it and residue 130. The mechanism for this cleavage and its relation to iodination are not clear. Reduction of iodinated Tg has produced hormone-rich N-terminal peptides of 10–30 kDa in all species studied. Gregg et al. (22) have localized these cleavages in reduced bTg to precede residues 81 and either 234 or 235. The cleavage we report here following Val129 appears different in type and location. Marriq et al. (25) also reported a cleavage between residues 129 and 130 in a CNBr-generated fragment of bTg. Although we do not discount the possibility that the break we describe occurs simultaneously with iodothyrosyl coupling at that site, we think it more likely that the formation of DHA with the loss of the phenyl ring at the time of coupling makes the 129–130 bond susceptible to breakage during subsequent experimental manipulation. We do not have evidence that this cleavage occurs in the thyroid in vivo. An N-terminal peptide comprised of residues 1–129 has not been reported in reduced Tg after various degrees of iodination. Gavaret et al. (2) proposed that instability of the peptide bond associated with the DHA residue could lead to its hydrolytic cleavage and the transformation of DHA to Pyr at the N terminus of the resultant peptide. Our mass spectral data favor the presence of Pyr rather than DHA in the donor peptides, at least by the time it was isolated and studied. Our inability to sequence the trypsic peptide containing residues 130–133, with the putative donor at 130, is consistent with Pyr blocking the Edman degradation. The work of Gentile et al. (12) suggests

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**Fig. 8. Analysis of Pyr-containing fraction (Peak C, Fig. 7), from Tg II. A, capillary HPLC separation; B, Electron spray-mass spectrometry spectrum of peptide 1 from panel A.** The measured molecular mass of the intact peptide is 1981.6 daltons. A fragment of this peptide is also present, representing a loss of 43 daltons.

**Fig. 9. Endo-Glu-C peptide residues 129-146 and its fragments.** A, theoretical peptide (22); B, 1981.6 dalton peptide; C, 1608.7 dalton peptide obtained after trypsin digestion, and its lost C-terminal tripeptide.
that cleavage of the peptide bond and the accompanying transformation of DHA to Pyr at the donor site are not universal consequences of the iodotyrosyl coupling reaction because they reported intact DHA at residue 1375 in bTg within a peptide comprising residues 1366–1381, as identified by mass spectrometry.

In this study, we concentrated on the N-terminal region of bTg because it contains the most important hormonogenic site (Tyr\(^5\)) of Tg and can be isolated by chemical reduction after iodination. Most of the N terminus was in fractions III and IV (Fig. 3 and Table I), which together contained similar portions of the total \([^{14}C]\)Pyr of Tg and of its total labeled hormone. We did not locate the \([^{14}C]\)Pyr in fraction IV, but suspect at least some was at residue 130 because this fraction contained appreciable amounts of 22 kDa on autoradiography (Fig. 3, insert); also FIV would be expected to include a residual peptide encompassing residues 81–234 after cleavage of the 22 kDa to produce an \(-10\) kDa, as shown in Fig. 6 (22).

Our previous studies showed Tyr\(^5\) and Tyr\(^{130}\) to be the only important early iodination sites in the first 240 residues of hTg (9), which is strongly homologous to bTg, although Xiao, et al. (26) did not find early iodination at Tyr\(^{130}\) in their in vitro system. Several reports suggest that the N terminus is sufficient by itself to form T\(_4\); for example, goitrous Dutch goats, with a hereditary defect leading to termination of Tg message transcription at residue 296, were still able to synthesize sufficient hormone for euthyroidism (27). In addition, Tyr\(^{130}\) and Tyr\(^5\) are prominently involved in hormone synthesis in several experimental models (16, 17, 28) although the applicability to in vivo conditions is uncertain. We believe these considerations make Tyr\(^5\) the most likely acceptor for the donated iodotyrosyl from position 130. Further study of hormone formation in the remainder of the Tg molecule is necessary to match donors and acceptors definitively.

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