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Efficient thyroid hormone formation by in vitro iodination of a segment of rat thyroglobulin fused to Staphylococcal protein A

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A polypeptide of 224 amino acids from the C terminus of rat thyroglobulin fused to Staphylococcal protein A (TgC 224), containing 3 tyrosines which have been shown to be hormonogenic in vivo (Tyr-2555, -2569 and -2748), forms thyroid hormones with relatively high efficiency upon in vitro enzymatic iodination using, most likely, the hormonogenic Tyr-2555 and Tyr-2569. Acetylcholinesterase, which has sequence and structural homology with the C terminus of the thyroglobulin molecule and bovine serum albumin, used as control proteins, formed thyroid hormones with lower efficiency. These results validate our experimental approach to define the structural requirements for thyroid hormone formation using thyroglobulin fragments.

Thyroglobulin; Thyroid hormone; Iodination; Hormonogenic site; HPLC fractionation

1. INTRODUCTION

The size of thyroglobulin represents a serious handicap for understanding the mechanism of biosynthesis of thyroid hormones by conventional techniques. Recently, thanks to the availability of recombinant DNA technology, the primary sequence of Tg from various species has been determined [1–3]. It became apparent that the structure of Tg has been well preserved during evolution in vertebrates, suggesting that it is well fitted for an efficient thyroid hormonogenesis, as shown earlier from in vitro iodination studies [4–6]. Still, the size of the Tg molecule seems to be out of proportion with the relatively very low yield of hormones (3–4 residues/molecule) restricted mainly to 4 hormonogenic sites [7–9] located in both the amino and the carboxy terminus, though an additional site has been recently reported in the middle of the molecule [10].

The presence of small, abnormal, Tg antigens in the hereditary goitre of the Afrikander cattle [11] and the Dutch goat [12], containing the N-terminal hormonogenic site could be responsible for the euthyroid state of the former and also for the correction by iodide administration of the hypothyroidism of the latter goitrous animals. This probably indicates that this site, which plays a very important functional role in the full-size Tg molecule [13] is also very important in the abnormal Tg [14]. It could eventually serve as a functional hormone precursor even out of the context of the entire Tg molecule as it has been suggested on the basis of the absence of sequence homology among different hormonogenic sites [15]. This evidence was taken as an indication that the different sites evolved independently and, consequently, that they could also function independently from each other. Thus, although the size of the Tg molecule could have evolved for an efficient synthesis of thyroid hormones and the storage in non-dischargeable form of iodine which is scarce in the earth's crust [6], it is also possible that the whole molecule is not directly involved in thyroid hormone biosynthesis. In order to investigate this possibility and also to define the structural requirements of the Tg molecule, we have synthesized [16] a construct of 224 amino acids from the C-terminal end of rat Tg (TgC 224) comparing its efficiency of thyroid hormone formation upon in vitro iodination with that of iodine poor human Tg, acetylcholinesterase (ACHE), which has sequence and structural homology with the C-terminal end of Tg but lacks its hormonogenic Tyr residues [17], and bovine serum albumin (BSA).

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2. MATERIALS AND METHODS

2.1. *Plasmids*

A thyroglobulin cDNA fragment, encoding for the last 224 amino acids at the carboxy terminus (position 2232–3018 in the sequence in Ref. [3]) and containing the 3′ untranslated region, was excised from plasmid pRT57 (3) with *Eco*RI and *Pst*I and cloned in the corresponding sites in the vector pERAT 316. In the resulting construct (pUM3)

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the thyroglobulin coding sequence is fused, in frame, with the region coding for Staphylococcal protein A. pUm3 was introduced into *E. coli* RR1 harbouring the low copy plasmid pNF2690 and the synthesis of the hybrid protein (TgC 224) was induced as described [18].

2.2. *Proteins*

Thyroglobulin. Iodine poor human Tg (660 kDa, 0.01% I; < 1 atom I/molecule) was purified and lyophilized as previously described [19]. For numbering the Tyr residues in TgC 224 we have followed the same as in reference [1] for bovine Tg.

Acetylcholinesterase (ACHE, 300 kDa) from electric eel and bovine serum albumin (BSA, 60 kDa) were from Sigma.

TgC 224. Extracts from induced bacterial cultures harbouring the pUM3 plasmid were prepared by sonication in a buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM PMSF (buffer A). The cell extract was centrifuged, the supernatant was made 50% in ammonium sulphate and the precipitated proteins were dialyzed against buffer A. Purification of the hybrid protein was achieved by affinity chromatography. CNBr activated Sepharose-4B (Pharmacia) was coupled to rabbit anti-Tg Ig-G according to the manufacturer's instructions. The dialyzed bacterial extract was cycled on the column three times. The column was washed sequentially with buffer A. Each wash was carried out until the A_{280} of effluent was <0.01. The bound proteins were then eluted with 0.5 M acetic acid and adjusted to pH 3.5 with ammonium acetate. The protein peak was pooled, dialyzed against 50 mM Tris-HCl pH 7.5 and stored at −20°C.

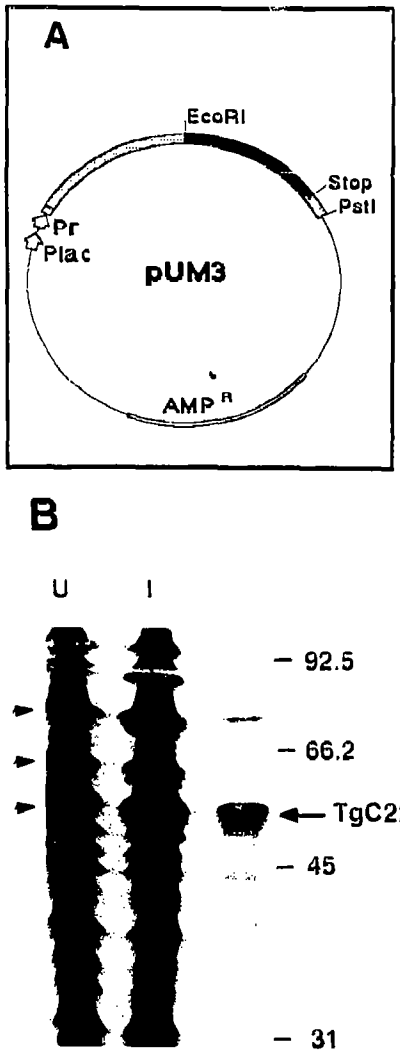

Fig. 1. Panel A, the structure of the pUM3 plasmid is shown. Empty box, NH_2 terminus of cro; stippled box, protein A coding region; black box, Tg coding region; lightly stippled box, Tg 3' untranslated region. Panel B, SDS-PAGE stained with Coomassie blue of crude extracts from uninduced (U) or induced (I) *E. coli* RR1 containing the pUM3 plasmid and the purified TgC 224 protein (P). Arrowheads indicate induced proteins. The position of TgC 224 and of molecular weight markers is indicated.

2.3. *Enzymatic iodination*

The various proteins were iodinated in vitro using lactoperoxidase and glucose-glucose oxidase as previously described [19]. The iodinated proteins were separated from the excess iodide and reagents by filtration through ECONOPAC 10 DG (Bio-Rad) disposable columns in 0.01 M Tris-HCl, 0.001 M MMI, pH 8.5.

2.4. *Reduction, alkylation and trypsin digestion*

The iodinated TgC 224 was reduced, alkylated and digested with TPCK trypsin (Sigma, 5% w/w) in 1% ammonium bicarbonate, 0.01% SDS for 16 h at 37°C, stopping the reaction with trifluoroacetic acid (TFA, Merck) and lyophylization as previously described [20].

2.5. *HPLC fractionation of tryptic digests and sequencing of purified peptides*

The tryptic peptides from iodinated TgC 224 were fractionated by HPLC on a UltrapackTM Octyl C_8 reverse-phase column (Beckman). The column was run at 0.5 ml/min and was initially equilibrated with 95% phase A (0.7% ammonium bicarbonate containing 5% acetonitrile) 5% phase B (100% acetonitrile) at room temperature; eluted over 130 min with a linear gradient of 5 to 40% phase B, then over 10 min to 100% phase B. They were further purified using a TFA-acetonitrile gradient. The column was equilibrated with 80% phase A (water, 0.09% TFA, pH 2.0), 20% phase B (water, 90% acetonitrile, 0.09% TFA) and eluted over 15 min with 20% phase B, then over 275 min from 20 to 45% phase B and then over 30 min from 45 to 100% phase B. The column was monitored with a photodiode array detector and a NEC APC IV computer, by counting for ^{125}I and by distribution of ^{125}I among iodoamino acids after pronase digestion and paper chromatography of most HPLC fractions (0.5 ml fractions) as previously described [20]. The purified peptides were sequenced either in a Beckman or in a Knauer modular liquid-phase protein sequencer equipped on line with a Knauer phenyl thiohydantoin-amino acid analyzer. The PTH derivatives were identified and quantified on a reverse phase HPLC system based upon a C_{18} column and gradient elution with 85% 6.5 mM sodium acetate, 15% acetonitrile, adjusted to pH 4.77 and 100% acetonitrile. Sequences were performed in the presence of polybrene. An aliquot from the PTH-derivatives obtained in each sequence cycle were counted for ^{125}I.

3. RESULTS

The plasmid pUM3 (Fig. 1A) allows the synthesis, in *E. coli*, of a rat thyroglobulin segment fused to Staphylococcal protein A (TgC 224). The hybrid protein shows the expected molecular weight (56 kDa) and can be readily purified on an anti-Tg antibody-Sepharose 4B column (Fig. 1B). TgC 224 accumulates in the overproducing strain as a partially soluble protein, as it distributes in almost equal parts between pellet and supernatant (data not shown). Only the soluble fraction was used in this study, to avoid the use of denaturing agents which would be necessary to solubilize the precipitated fraction. The bands of lower molecular weight, present in our purified preparations (Fig. 1B), presumably represent degradation products, as they are

Table I

Degree of iodination, [^{125}I]iodoamino-acid distribution and number of residues formed per molecule by in vitro enzymatic iodination of iodine poor (0.01%) thyroglobulin, TgC 224, acetylcholinesterase and BSA (for more details see text)

Protein	Atoms I/mol	[^{125}I]iodoamino-acid distribution				Residues/mol			
		(%DIT)	(%MIT)	(%T4)	(%T3)	DIT	MIT	T4	T3
Tg	17.5	34.5	31.1	19.5	1.5	3.1	5.4	0.85	0.09
TgC 224	17.8	37.3	28.4	8.5	1.0	3.3	5.0	0.38	0.06
ACHE	22.8	32.0	30.4	3.9	0.6	3.6	6.9	0.22	0.04
BSA	17.0	52.5	17.8	5.6	0.4	4.4	3.0	0.27	0.02

able to bind anti-Tg antibodies (data not shown). The contaminant of higher molecular weight, the nature of which is unknown, represented less than 10% of the total protein. To assess the efficiency of thyroid hormone formation in TgC 224, we compared it with a full-size human thyroglobulin, with ACHE, and with BSA. Table I shows that although Tg is the most efficient for thyroid hormone formation, TgC 224 is not only quite efficient but somewhat specific since it forms more than twice as much T_4 than ACHE and almost twice as much as BSA, as more clearly summarized in Fig. 2.

In order to assess where the T_4 formed by TgC 224 was located, and to find out whether it is formed preferentially at the same Tyr residues which have been demonstrated to be hormonogenic in vivo, the iodination reaction was scaled up. 7 mg of TgC 224 was iodinated in vitro with 1.25×10^{-4} M potassium iodide labeled with radioiodide (S.A. = 1 μCi/μatom) at a level of 6.5 atoms of iodine per molecule. The iodoamino acid distribution of the iodinated protein was: 31.6% diiodotyrosine (DIT), 58.1% monoiodotyrosine (MIT), 3.5% T_4 and 2.7% triiodothyronine (T_3). The tryptic peptides from iodinated TgC 224 were separated by successive HPLC gradients. Fig. 3 shows the ^{125}I profile of the TFA gradient in the left panel and the ^{125}I cpm found as T_4 in each fraction in the right panel. The T_4-rich peaks numbered 1–6, contributing to as much as 57.5% of the total T_4 formed, though also containing a high proportion of iodotyrosines, were submitted to sequencing. As shown in Table II, four of them (36.7% of the T_4 formed by TgC 224) gave the same main and contaminating sequence corresponding to Tg hormonogenic sites at Tyr-2555 and Tyr-2569, respectively [21,22]. The PTH derivatives were counted for ^{125}I (data not shown) and though peaks were seen in position 18 and 2, corresponding to the hormonogenic Tyrs included in the main and the contaminating sequence positions (positions 2555 and 2569, respectively), there was a higher peak in position 8 and 9, corresponding, most likely, to the iodotyrosines present in the peptides.

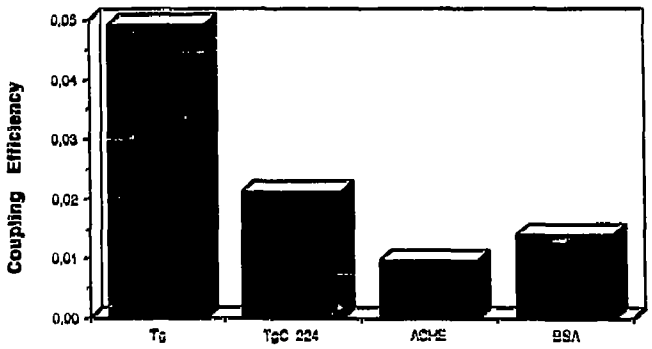

Fig. 2. Coupling efficiency corresponding to the data in Table I, measured as the number of T_4 residues formed per atom of iodine bound per molecule of Tg, TgC 224, ACHE and BSA.

4. DISCUSSION

Although the primary sequence of the Tg molecule from various species has recently been made available [1–3], little progress has been made toward an understanding of the process leading to the formation of thyroid hormones. In particular, no indication has been obtained on two key aspects of the hormonogenic process, i.e. the selection of some specific tyrosines and the requirements of the different parts of the molecule for an efficient utilization of iodine.

Our approach to elucidate the molecular basis of the selection of a specific set of tyrosines for thyroid hormone formation has been to synthesize defined segments of the Tg molecule containing hormonogenic sites [16] and to study their efficiency of thyroid hormone formation in vitro. If the same tyrosines are used in our simple model system as in vivo, then a mutagenic analysis could be undertaken to understand the structural requirements for this highly specific process. We have obtained a 224 amino acid rat Tg fragment fused to Staphylococcal protein A (TgC 224). This segment of Tg contains a cluster of tyrosines among which three (Tyr-2555, -2569 and -2748) have been shown to be hormonogenic in vivo and in vitro [21,22]. To validate our construct we compared its ability to form thyroid hormones in vitro with that of the full-size Tg molecule. Two negative controls were used: (i) ACHE, which shows a strong sequence homology with the C-terminal end of Tg although it lacks the hormonogenic Tyr residues [17]; (ii) BSA, because it is known that albumin, in the absence of the normal substrate Tg, as is the case of some congenital goitres [14], becomes iodinated and

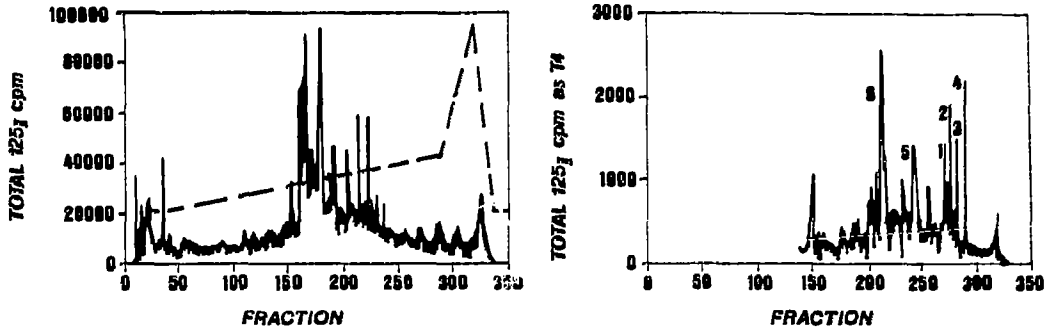

Fig. 3. HPLC fractionation of tryptic peptides from in vitro iodinated, reduced-alkylated TgC 224. Left panel, total ^{125}I cpm per fraction. Right panel, percent of the total T_4 formed by TgC 224 found in the various fractions. The figures within the arrows indicate the percent of total T_4 found among those fractions. Numbers by the sides of the peaks indicate those that were submitted to sequencing. For more details see text.

forms some thyroid hormones. The fact that TgC 224, which is less than 10% the size of Tg, forms, for comparable degrees of iodination, almost half as much thyroid hormone as Tg suggests that TgC 224 is quite efficient especially when considering that BSA formed about half as much and ACHE less than half. It was then very crucial to determine whether the T_4 formed by TgC 224 was located within the Tg or the protein A portion. All The T_4-rich peptides sequenced not only belonged to the Tg portion but they were contained within Tg hormonogenic sites. Though not directly demonstrated, due to the scarcity of material, they most likely correspond to the hormonogenic Tyr-2555 and Tyr-2569. This essential point and the subsequent mutagenic study are under progress in a similar construct (TgC) which does not have the protein A portion and synthesizes thyroid hormones with similar efficiency as TgC 224 [23]. Recently Rawitch et al. [24] have reported a pulse-chase study with human Tg in which Tyr-5 was found to behave as the main hormonogenic site while Tyr-2555 behaved as a donor since the label found on it with the pulse was disappearing during the chase. Since the experimental conditions differ significantly it is difficult to interpret this difference at the moment.

The present results show that a small Tg fragment, fused to Staphylococcal protein A produced in *E. coli*, forms a fair amount of thyroid hormone, in vitro, most likely using the Tg hormonogenic Tyr-2555 and -2569 and indicate that the approach illustrated in this paper may give important information on the structural requirements for hormonogenesis in the Tg molecule.

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Table II

Percent of DIT plus MIT, T_4 and percent of the total T_4 formed by TgC 224 corresponding to the ^{125}I-tryptic peptides 1–6 from right panel of Fig. 3 and sequences of the main and contaminating peptides obtained, aligned as in sites no. 2555–2569 of rat TG

				Sequence	
Peptide	%DIT+MIT	%T4	% of total T_4	Main	Contaminating
1	80.3	9.3	7.4	ILAAAIWYYSLEHSTDDYASFSR	(DYFII-PIVN)
2	79.3	12.2	10.0	ILAAAIWYYSLEHSTDD	(DYFII-PIVN)
3	74.3	16.3	7.9	ILAAAIWYYSLEHSTDDYASFSR	(DYFII-PIVN)
4	78.2	12.2	11.4	ILAAAIWYYSLEHSTDDY	(DYFII-PIVN)
5	80.2	8.8	7.4		
6	70.0	9.8	13.4		

Rat TG: ILAAAIWYYSLEHSTDDYASFSR——RDYFIICPIVN

Tyr no. 2555 2569

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