Exposure of thyroxine residues in human thyroglobulin

Two-site binding studies

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Human thyroglobulin (Tg) could be adsorbed through one of its thyroxine (T_4) residues by either of two T_4 -binding antibodies which had been covalently attached to Sepharose-CL4B. The antibodies used were (i) a purified human autoantibody specific for a T_4 -containing epitope in human Tg, or (ii) a rabbit antibody raised against T_4 conjugated to bovine albumin side chains. Tg adsorbed by either immobilized antibody could then itself adsorb either type of antibody free in solution on to a further T_4 residue. At least two T_4 residues in human Tg are therefore sufficiently exposed to interact with T_4 -binding antibodies. Furthermore, these T_4 residues are sufficiently far apart to allow the binding of two immunoglobulin molecules simultaneously. Previous observations of a marked preference by human autoantibodies for one of the T_4 -containing epitopes in Tg therefore reflect a higher binding energy with that epitope rather than an inability to interact with others. The T_4 -containing epitope which preferentially reacts with human Tg autoantibodies must therefore have a distinctive topography.

Thyroglobulin (Tg) contains the amino acid 3,3',5,5'-tetraiodothyronine (thyroxine, T_4) in peptide linkage within its backbone structure. Up to eight such T_4 residues may be found (Marriq *et al.*, 1980), but a mean of approximately four residues per molecule is more usual (e.g. Byfield *et al.*, 1982). It may be assumed that these residues are at least near the surface of the protein if not on it, for they are formed by an enzyme-catalysed post-translational modification. At least one T_4 residue is on the surface, since T_4 -binding antisera react with Tg through such a group (Pearce *et al.*, 1981). Further studies led to the conclusion that two human autoantibodies with different properties had a preference for one particular T_4 -containing site in human Tg (Byfield *et al.*, 1982). These findings suggested local structural differences around the various T_4 residues. Those conclusions, however, were drawn from studies made in competitive-type binding experiments using limiting quantities of antibody and with [125 I] T_4 as a tracer. It was thus possible that T_4 -containing sites in Tg with a binding energy significantly less than

that of [125 I] T_4 would not have been detected, despite their being accessible to antibodies. The present study was therefore undertaken to clarify whether the observed preference of the autoantibodies was real in the face of a choice of T_4 residues or whether the reaction was confined to one residue because others were physically inaccessible to immunoglobulins, either before or after the first immunoglobulin had bound.

Methods

Preparation of human thyroglobulin

Human thyroid tissue was obtained 36 h *post mortem* and frozen at -30°C until use. The tissue was thawed, finely chopped and extracted for 2 h with sodium phosphate buffer (0.05 mol/litre), pH 7.4, containing 6-aminohexanoic acid (0.05 mol/litre) (3 ml/g of tissue). The extract was centrifuged at 300g for 15 min and the clear red supernatant chromatographed in 5 ml portions on a column (1.5 cm $\times$ 75 cm) of Sepharose-6B. The Tg peak was located by a radioimmunoassay for T_4 , which cross-reacts with Tg, by protein-concentration measurements (Lowry *et al.*, 1951) and in some runs by u.v. absorption. Central regions of the peak were pooled, concentrated by pressure dialy-

Abbreviations used: Tg, thyroglobulin; T_4 , thyroxine (3,3',5,5'-tetraiodothyronine); T_3 , 3,3',5-tri-iodothyronine; r T_3 , reverse T_3 (3,3',5'-tri-iodothyronine); T_2 , 3,5-di-iodothyronine.

sis and stored at -30°C until required. The thyroglobulin yield was 114 mg from 6.7 g of tissue.

The T_4 content of the Tg preparation was 4.1 residues/molecule (determined by radioimmunoassay of a total enzymic digest of the protein).

Purification of a human T_4 -binding autoantibody (hT_4TgAb)

Detailed studies of this autoantibody, which is mainly directed against a T_4 -containing epitope in human thyroglobulin, have been published (Pearce *et al.*, 1981; Byfield *et al.*, 1982; Byfield & Bond, 1982). The antibody cross-reacts with iodothyronines free in solution but shows a high specificity for T_4 . By using [^{125}I] T_4 as tracer, cross-reactivities with the antiserum were: T_4 , 1.0; T_3 , 0.01; rT_3 , 0.004.

The serum contained a minor component of Tg antibodies which did not bind iodothyronines. Therefore, for these studies, the iodothyronine-binding component of the autoantibody was selectively extracted. Serum (3.5 ml) was first stripped of endogenous iodothyronines by eight passes through a 3.5 ml column of Amberlite CG 400 ion-exchange resin. It was then passed through a column (3.5 ml) of T_2 -Sephacrose-CL4B, which adsorbed all of the T_4 -binding antibody activity in the sample. After washing the gel with 5 vol. of 0.1 M-barbitone buffer, pH 8.6, the T_4 -binding antibody was eluted by 3 vol. of 0.1 M- NH_3 in 20% (v/v) ethanol. Recovery of antibody activity in six preparations was 72–100% (mean 86.5%).

Synthesis of a T_4 -albumin conjugate

T_4 was covalently attached to human serum albumin by a carbodi-imide condensation method (Burke & Shakespeare, 1975). Human serum albumin (50 mg) and 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide hydrochloride (100 mg) were dissolved in 15 ml of water and cooled to 0°C . T_4 (5.2 mg) was dissolved in 5 ml of dimethyl-formamide/water (1:1, v/v) with the aid of a few drops of 1 M-NaOH. A trace amount of [^{125}I] T_4 was added to allow the calculation of the T_4 content of the final product. This solution was cooled to 0°C and added slowly with stirring to the albumin solution, maintaining the pH at 7.5 ± 0.5 with 1 M-HCl. This mixture was then left with stirring overnight at 4°C . Small, variable amounts of precipitate formed and were removed by centrifugation. The opalescent solution was then extracted four times with butan-1-ol or until no more radioactivity was extracted into the organic phase. Butanol in the aqueous phase was extracted with diethyl ether and the T_4 -albumin conjugate solution was chromatographed in 10 ml portions on a column (1.6 cm $\times$ 28 cm) of Sephadex G-50 in 0.1 M- NH_4HCO_3 . The protein peak was pooled and

freeze-dried. The protein (Lowry *et al.*, 1951) and radioactivity contents of this product allowed the determination of the mean amount of T_4 (in mol) attached/mol of albumin. The sample used in the present work contained 1.77 mol of T_4 /mol of albumin.

Affinity adsorbents

Sephacrose-CL4B was activated with tosyl (toluene-*p*-sulphonyl) chloride and used as described by Nilsson & Mosbach (1980).

One sample of the human T_4 -binding antibody hT_4TgAb purified as described above and equivalent to the yield from 0.88 ml of serum had a protein content of 1.25 mg. It was dissolved in 2.5 ml of 0.1 M- NaHCO_3 /0.5 M-NaCl and mixed with 2.5 ml (settled volume) of tosyl-Sephacrose-CL4B for 2 h at room temperature. All the T_4 -binding activity was immobilized on the solid phase.

The rabbit T_4 -binding antibody (rT_4Ab) was a commercially available immunoglobulin fraction (Miles Laboratories, Stoke Poges, Bucks., U.K.) raised against T_4 coupled to bovine albumin. A 100 μl portion of the solution was added to 30 ml of 0.1 M- NaHCO_3 /0.5 M-NaCl and allowed to react with 24 ml (settled volume) of activated gel for 2 h at room temperature, when 47% of the T_4 -binding activity, determined as described below, had been immobilized on the gel.

The T_2 (45 mg) was dissolved in 10 ml of 0.1 M- NaHCO_3 buffer, pH 10, containing 0.5 M-NaCl and allowed to react with 1.0 ml (settled volume) of activated gel for 2 h at room temperature. A trace amount of [^{125}I]tri-iodothyronine was included in the mixture to enable the rough estimation of the amount of T_2 coupled to the gel by radioactivity measurements. On this basis, 32.4 mg of T_2 was immobilized, giving a concentration of 6.2 $\mu\text{mol/ml}$ of gel.

After coupling, each gel was exhaustively washed with the buffer used in coupling and with 0.1 M-sodium acetate/1.0 M-NaCl, pH 4.0.

Two-site binding studies

Small, matched columns (0.5 ml) of hT_4TgAb - and rT_4Ab -Sephacrose-CL4B gels were equilibrated with 0.1 M-barbitone buffer, pH 8.6. A portion (0.5 ml) of a solution of human Tg or T_4 -albumin in the buffer was passed through the gel. Unbound protein was washed out with buffer until no protein could be detected in the effluent by the Lowry method.

A solution of either hT_4TgAb (purified as described above) or rT_4Ab in buffer (3×0.5 ml) was then passed through each column, followed by 4×0.5 ml portions of buffer, 0.5 ml fractions of the

effluent being collected. The amount of antibody in each fraction of the effluent was determined by measuring the amount of [¹²⁵I]T₄ bound by a known volume of the effluent and comparing it with the amount bound by dilutions of the antibody solution originally applied to the column. The results were expressed as μl equivalents of the original antibody solution. Control experiments were performed in which solutions of antibody were passed through columns of unsubstituted Sepharose-CL4B and, in the case of the human variety only, a column of antibody-Sepharose-CL4B which had not been exposed to Tg.

A sensitive radioimmunoassay for human Tg which does not cross-react with T₄ or T₄-containing epitopes in Tg was used to check that no Tg was stripped from the columns by the soluble antibodies flowing through. None was found in any experiments.

Results

The feasibility of this approach to examining the exposure of T₄ side chains in proteins was first examined in a model system.

A human albumin derivative which contained a mean of 1.77 molecules of T₄ per molecule was selected. Four experiments were performed in which increasing amounts of T₄-albumin from 63 to 500 μg were applied to matched columns of immobilized rT₄Ab. In each case all of the modified protein was adsorbed by the solid-phase antibody. A solution of rT₄Ab was then passed through each column, followed by buffer washes as described in the Methods section. Antibody in solution was adsorbed on to the now-immobilized T₄-albumin as indicated by reduced recoveries (Fig. 1), and the amount adsorbed was related to the quantity of T₄-albumin held in the column.

Fig. 1. Simultaneous reaction of rT₄Ab at two sites on T₄-substituted albumin

Various amounts of T₄-substituted albumin (63, 125, 250, 500 μg) were adsorbed on a series of matched columns (0.5 ml) containing immobilized rT₄Ab. The protein was held fast to the antibody by one of its T₄ haptens. The ability of the immobilized bivalent ligand to adsorb antibody activity from 1500 μl of rT₄Ab solution was tested as described in the Methods section. The four elution profiles record the amounts of rT₄Ab recovered in fractions (500 μl) of the effluent from each column. The total amounts of this T₄-binding activity recovered from the columns decreased (266, 201, 139, 71 μl equivalents) as the quantity of T₄-substituted albumin present increased from 63 to 500 μg.

Table 1. Amounts of bivalent antigens adsorbed by the immobilized antibodies and amounts of soluble antibody subsequently bound by the adsorbed antigens

Abbreviation used: n.d., not determined.

Immobilized antibody	Bivalent antigen			Soluble antibody	
	Type	Loaded (μg)	Adsorbed (μg)	Type	Adsorbed (%)
hT ₄ TgAb	Tg	735	178	hT ₄ TgAb	86
	Tg	790	n.d.	rT ₄ Ab	88
	T ₄ -albumin	210	190	hT ₄ TgAb	96
	T ₄ -albumin	195	195	rT ₄ Ab	95
		0	0	hT ₄ TgAb	17
rT ₄ Ab	Tg	735	319	hT ₄ TgAb	91
	Tg	790	328	rT ₄ Ab	83
	T ₄ -albumin	210	210	hT ₄ TgAb	97
	T ₄ -albumin	195	195	rT ₄ Ab	94
		0	0	hT ₄ TgAb	30
None		0	0	rT ₄ Ab	11

This experiment clearly showed that this bivalent ligand, presenting two T_4 moieties to the solvent, reacted first with the immobilized T_4 -binder and could subsequently react with rT_4Ab and adsorb it from solution. Thus any protein which can do this while being held fast by a T_4 side chain must have two or more T_4 residues exposed and accessible to immunoglobulins as follows:

This method was then applied to the case of human Tg. Two types of solid-phase antibody were used: the rabbit antibody against T_4 conjugated to bovine albumin (rT_4Ab) and the purified human autoantibody specific for a T_4 -containing site in Tg (hT_4TgAb). Human Tg was extracted from solutions passing through columns of either type of solid-phase antibodies (Table 1) and bound via a T_4 residue. After the introduction of Tg the columns were washed with buffer until no more protein (Tg) was detected in the effluent. Control experiments using T_4 -albumin as the bivalent ligand were performed at the same time (Table 1).

Recovery of antibody from blank columns was 70–89% of the amount applied (Table 1 and Fig. 2). However, when human Tg was immobilized in the columns, recovery was substantially decreased to 9–17%. When T_4 -albumin was used as the bivalent ligand, recovery of antibody was a little less at 3–6% of the total applied.

Thus the same effect was observed irrespective of whether the Tg had been immobilized on either the rabbit or the human T_4 -binding antibody, despite their being raised against very different immunogens. At least two T_4 side chains in human Tg were therefore available for simultaneous reaction with immunoglobulins.

No Tg could be detected by radioimmunoassay on the effluents from the adsorbent columns. The reduction of [^{125}I] T_4 -binding capacity in these effluents therefore reflected a decrease in antibody amount and not competition from Tg stripped from the affinity gel.

Discussion

The model system using the bivalent T_4 -albumin conjugate, immobilized rT_4Ab and soluble rT_4AB confirmed the validity of this two-site binding method to examine the exposure of T_4 side chains on the surface of a protein.

Human Tg was extracted from solution by both hT_4TgAb and rT_4Ab attached to Sepharose. The interaction was presumably through a T_4 -residue side chain in Tg. Confusion due to the presence in the patient's serum of Tg antibodies against non- T_4 -containing epitopes (Pearce *et al.*, 1981) was eliminated by using the purified T_4 -binding component. Antibodies in rT_4Ab reacting with non- T_4 -containing epitopes on human Tg would not be expected. Irrespective of the type of solid-phase antibody used to immobilize the Tg, the soluble T_4 -binding antibodies of rabbit or human origin were subsequently adsorbed on to the Tg. This second binding reaction must have been through a T_4 residue and demonstrates that at least two T_4 side chains in human Tg are sufficiently exposed to interact with immunoglobulins. Furthermore, they must also be so placed that there is no steric hindrance to the simultaneous binding of two immunoglobulins.

At first sight these conclusions are at variance with our previous results (Byfield *et al.*, 1982), where antibodies were shown to have a strong preference for binding to one particular T_4 residue in Tg. It is, however, only a matter of degree. The earlier experiments were done in a competitive system with small, limiting quantities of antibody (much less in molar terms than the quantity of Tg). [^{125}I] T_4 was used as a tracer and only the one T_4 residue in Tg with an affinity for antibodies comparable with that of T_4 was detected. Other T_4 residues with significantly lower affinities would have escaped detection.

In the two-site binding system, Tg was bound to the solid-phase antibody on a 1:1 molecular basis (or, at worst, on a 1:2 basis if the two binding sites on one immunoglobulin molecule each bind one Tg

Fig. 2. Simultaneous reaction of T_4 -binding antibodies at two sites on human Tg

Four experiments were performed using the four possible combinations of hT_4TgAb and rT_4Ab where one was immobilized and one dissolved. Tg was allowed to bind through a T_4 residue to the immobilized antibodies as described in the Methods section. To examine the ability of the bound Tg to adsorb T_4 -binding antibodies, solutions (1500 μ l) of either hT_4TgAb (a, c) or rT_4Ab (b, d) followed by 2000 μ l of buffer were passed through columns (0.5 ml) containing Tg bound to either hT_4TgAb (a, b) or rT_4Ab (c, d) covalently attached to Sepharose-CL4B. Fractions (500 μ l) were collected. Control experiments with T_4 -substituted albumin or no ligand were also carried out. For quantities of antigens adsorbed in each experiment, see Table 1. The ordinates in each panel record the quantity of T_4 -binding activity detected in each fraction in experiments with no Tg bound ($\square$), with Tg bound ($\blacksquare$), with T_4 -substituted albumin bound ($\bullet$) and with columns of unsubstituted Sepharose-CL4B ($\circ$). In each experiment the presence of Tg caused a substantial decrease in T_4 -binding activity found in column effluents. The same effect was seen in the presence of the bivalent T_4 -substituted albumin.

molecule). Whether or not the immobilization was effected through the preferred T₄ residue is immaterial. Even a low-affinity T₄ residue would subsequently interact with the soluble antibodies, since there was no element of competition involved. Antibodies will, after all, accept inferior antigens if only they are available.

These studies show that human Tg contains at least two T₄ residues capable of simultaneously binding to immunoglobulins. The preferential binding of autoantibodies to one particular T₄ residue (Byfield *et al.*, 1982) is therefore due to a higher reaction energy with the epitope containing that residue. This discrimination is presumably associated with variations in the nature of structures surrounding the T₄ residues in the protein. Such variations have been predicted from the observation that individual T₄ residues in Tg are synthesized in an order that is controlled by the tertiary structure of the protein (Rolland *et al.*, 1973; Lamas *et al.*, 1974; Dème *et al.*, 1976; Gavaret *et al.*, 1977; Maurizis *et al.*, 1981). Confirmation has come from the recent determination of two different amino acid sequences around T₄ residues in porcine Tg (Marriq *et al.*, 1982). However, although the previous studies (Byfield *et al.*, 1982) indicated that the first T₄ residue to be formed was in the immunogenic site, it is not yet

possible to identify that site with a particular amino acid sequence.

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