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Thyroid Peroxidase as an Autoantigen in Hashimoto's Disease: Structure, Function, and Antigenicity

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ABSTRACT

Human thyroid peroxidase (TPO), is an important enzyme responsible for the biosynthesis of thyroid hormones and is a major autoantigen in autoimmune thyroid diseases (AITDs) such as the destructive Hashimoto's thyroiditis. Although the structure of TPO has yet to be determined, its extracellular domain consists of three regions that exhibit a high degree of sequence similarity to domains of known three-dimensional structure: the myeloperoxidase (MPO)-like domain, complement control protein (CCP)-like domain, and epidermal growth factor (EGF)-like domain. Homology models of TPO can therefore be constructed, providing some structural context to its known function, as well as facilitating the mapping of regions that are responsible for its autoantigenicity. In this review, we highlight recent progress in this area, in particular how a molecular modelling approach has advanced the visualisation and interpretation of epitope mapping studies for TPO, facilitating the dissection of the interplay between TPO protein structure, function, and autoantigenicity.

Introduction

Human thyroid peroxidase (TPO) is the major enzyme responsible for the biosynthesis of the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) [1]. TPO is a critical autoantigen and is a key antigenic target for autoantibodies (aAbs) in autoimmune thyroid disease (AITD), such as the destructive Hashimoto's thyroiditis and Graves' disease [2]. In AITD there is a breakdown in tolerance to a number of thyroid specific autoantigens and the generation of an autoantibody response. Autoantibodies against TPO, previously known as the thyroid "microsomal" antigen [2], are almost universally present in patients with active forms of AITD [3, 4].

Hashimoto's thyroiditis was the first recognised autoimmune disease and was first described over 100 years ago [5]. It is a classical example of AITD whereby an immune response is directed at the thyroid gland leading to the complete destruction of the thyroid (by $CD8^+$ T-cell infiltration) and reduced biosynthesis of critical thyroid hormones T_3 and T_4[6]. Thyroid hormones T_3 and T_4 affect the majority of body systems, with the symptoms and severity of disease ranging from neurological to physical manifestations that include hypertension, seizures, muscle degeneration, and diabetes [7]. The presence of high titer autoantibodies to TPO is a hallmark of disease and is frequently used as a diagnostic indicator [8, 9]. The exact mechanism by which thyroid damage is caused remains unknown, however, there is evidence to suggest that antibodies against TPO are responsible for the autoimmune destruction of thyrocytes [10], either by fixing complement or through cell-mediated cytotoxicity [11]. The precise system with which these antibodies cause damage is unknown [12]. Additionally, cases of AITD can occur without these autoantibodies being present, and transplacental passage of anti-TPO antibodies does not necessarily cause thyroid damage in the offspring [12–14].

* These authors contributed equally to this manuscript.

TPO is a 933 amino acid long transmembrane hemoprotein made up of several domains and is extensively glycosylated. There has been extensive study in mapping the epitopes of TPO given its clinical significance, however, with the lack of an experimentally derived structure the locations at which these autoantibodies bind and thus cause disease remains with no definitive answer [11]. A working knowledge of the structure of TPO as it relates to autoantibody recognition will help to understand the molecular pathologies of these AITDs.

TPO Biosynthesis, Maturation, and Trafficking

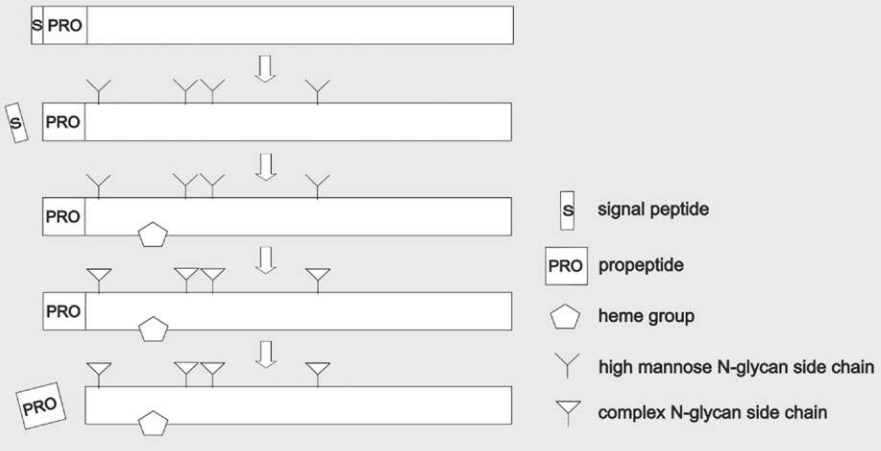
Upon translation of the TPO gene and processing in the endoplasmic reticulum (ER), the nascent polypeptide undergoes post-translational modifications that are critical to its catalytic function, including N-glycosylation and fixation of a prosthetic heme group (protoporphyrin IX) (▶ **Fig. 1**). The correct incorporation of the heme porphyrin ring is essential for the nascent protein's exit from the ER and indeed for protein functionality [15]. A reaction between the heme group and hydrogen peroxide allows for modification and covalent attachment of the heme group to TPO via an autocatalytic process [16]. Additionally, hydrogen peroxide allows for a more effective establishment of the two ester bonds between TPO and heme, leading to an increase in active TPO when in the presence of hydrogen peroxide [15]. In vivo hydrogen peroxide is provided by the dual oxidase (DUOX) family of proteins, which are also present in the thyroid follicular cell membrane and are closely associated with TPO [17, 18]. The N-terminal pro-peptide sequence is removed before it reaches the cell surface [19], where it resides as a type 1 transmembrane glycoprotein in the thyrocyte (thyroid follicular cell) membrane. However, only 2 % of full length TPO that is synthesised by cells reaches the cell surface with most of TPO retained in the ER, with misfolded forms of TPO (partially or completely misfolded) being degraded by the proteosome or proteases [20]. N-Linked glycosylation supports folding events and interactions between the nascent polypeptide chain and molecular chaperones [20]. Moreover, many active splice variants of TPO have been characterised contributing to the highly complex nature involved in its biosynthesis [21–23].

Biosynthesis of thyroid hormones occurs at the apical membrane of thyrocytes where tyrosine residues on thyroglobulin (Tg) are iodinated and subsequent iodotyrosyl residues on Tg are coupled to produce T_3 and T_4[1]. T_4 is principally carried in the blood by thyroxine-binding globulin enabling it to affect a large number of organs and organ systems. It is a critical hormone that is under tight regulation during proper thyroid function in normal human physiology. Pathologies resulting in thyroid dysfunction and loss of T_4 require hormone replacement therapy. The biosynthesis of T_4 by TPO requires the presence of free iodide, hydrogen peroxide (H_2O_2) as well as Tg that provides tyrosine substrates for iodination [24].

Interestingly, recent studies have shown that TPO is also expressed in extra-thyroidal tissues, that is, breast [25–27] and orbital [28, 29] tissues. More detailed analyses have demonstrated that breast TPO is effectively recognised by conformation-sensitive autoantibodies, undergoes N-glycosylation and heme incorporation [25, 26]. Although breast TPO exhibits similar properties to thyroid TPO, its expression level is significantly lower than in thyroid gland [25–27]. Further studies are needed to explain the role of TPO in extrathyroidal tissues. The most attractive hypothesis is that breast TPO is responsible for protective role of TPO autoantibodies in patients with breast cancer [25, 30, 31]. While, orbital TPO may be involved in beneficial modulating of immune response in patients with Grave's disease, and leads to decreased risk of clinical manifestation of Graves' ophthalmopathy [29, 32].

TPO Domain Architecture

TPO belongs to the family of human peroxidases together with lactoperoxidase (LPO), myeloperoxidase (MPO), and eosinophil peroxidase (EPO). These homologues of TPO share 48 %, 47 %, and 47 %



▶ **Fig. 1** TPO biosynthesis: High mannose oligosaccharide side chains and heme group are incorporated to the TPO translation product in the endoplasmic reticulum. Signal peptide cleavage occurs also in this compartment [like in myeloperoxidase (MPO)] or later during TPO transport to the cell surface (no data available). Complex N-glycan chains are added to the TPO polypeptide in the Golgi apparatus. The propeptide is removed after its transit through the Golgi apparatus but before it reaches the plasma membrane. Mature TPO forms dimers (not shown in this scheme), however, the localisation of dimerisation process in the cell is still unknown.

sequence identity with TPO, respectively. Sequence alignment of these proteins reveals conserved catalytic residues together with amino acids that are involved in the covalent linkage of the prosthetic heme that are critical for enzymatic function (▶**Fig. 2**). The full length TPO protein consists of 933 residues (107 kDa) and unlike the other human peroxidases is largely an extracellular protein anchored to the apical membrane of thyrocytes (thyroid follicular cell) by a transmembrane domain at its C-terminal end (residues 872–933) (▶**Fig. 2**). As mentioned before, TPO is synthesised with an N-terminal signal sequence and propeptide that are absent in the mature protein (residues 1–108), which is cleaved upon the protein leaving the ER. The TPO extracellular domain (ectodomain) consists of three regions that exhibit a high degree of sequence similarity to domains of known three-dimensional (3D) structure (▶**Fig. 3a**): the myeloperoxidase (MPO)-like domain (residues 142–738), complement control protein (CCP)-like domain (residues 739–795), and epidermal growth factor (EGF)-like domain (residues 796–846). These structurally elucidated homologues share 47 %, 35 %, and 54 % sequence identity with TPO domains, respectively.

Myeloperoxidase-Like Domain

Human TPO contains a conserved MPO-like domain (residues 142–738). The X-ray crystal structure of human MPO (▶**Fig. 3b**) revealed that it occurs as a homodimer that is linked covalently by a conserved intermolecular disulfide bond (Cys319) at the dimer interface. The overall fold of MPO is highly α-helical with little β-sheet. Each monomer has a central core of 5 helices and a prosthetic heme group that is covalently attached to Glu408 and Asp260. His261 is also essential for catalytic function and is also necessary for calcium binding, which in turn is involved in coordination of the heme group that is crucial for enzyme function. The calcium binding site is highly conserved across all animal peroxidases. In MPO and LPO, mutations that disrupt the calcium binding site result in peroxidases that are expressed in cells but not secreted as effectively as the wild type, and as such calcium may play a role in structure during secretion and biosynthesis [33]. Given the high degree of sequence similarity between TPO's MPO-like domain and MPO, including the conservation of the cysteine involved in disulfide linkage between monomers, it is likely that TPO dimerises via its MPO-like domain.

Complement Control Protein-Like Domain

The next region in TPO's ectodomain is its CCP-like domain (residues 739–795), also known as a Sushi domain. These domains contain short consensus repeats mostly found on complement control and adhesion proteins. The CCP-like domain is structurally based on the formation of three β-strands that are hydrogen bonded at one face and two separate β-strands at the other face, ultimately forming a β-sandwich arrangement of strands (▶**Fig. 3c**).

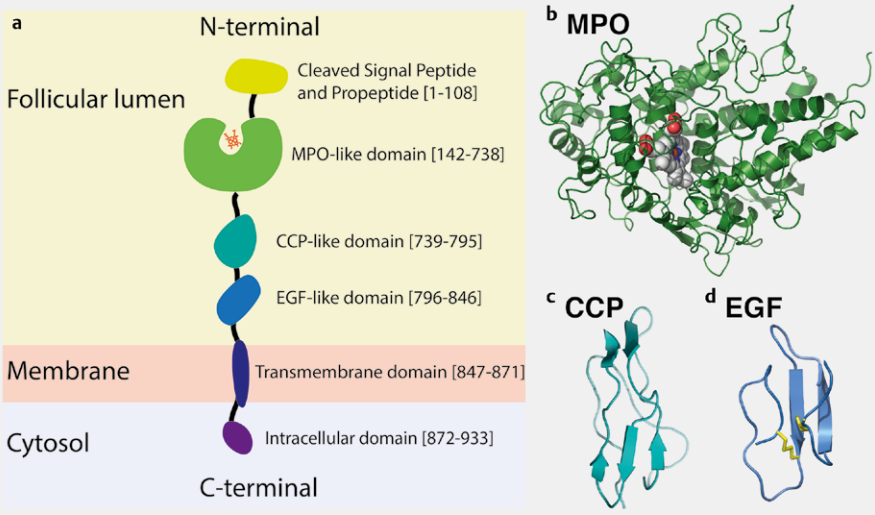
Epidermal Growth Factor-Like Domain

The membrane proximal region in TPO's ectodomain is the EGF-like domain (residues 796–846). This domain is highly evolutionarily conserved and found in the extracellular regions of membrane-bound proteins. The main characteristic of the EGF-like domain is the presence of 6 cysteine residues shown to form 3 disulfide bonds with an overall β-sheet fold followed by a short loop (▶**Fig. 3d**).

Structural Determinants of TPO Autoantigenicity

Knowledge of the TPO domain architecture, and particularly the high sequence homology shared between TPO's MPO-like domain and MPO (47 %), has allowed homology models of TPO to be con-

▶**Fig. 2** Sequence alignment of human myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), and thyroid peroxidase (TPO). Important catalytic residues are highlighted with red asterisks, and amino acids involved in the covalent link with the prosthetic group are in green asterisks. Glycosylation sites in MPO (asparagines, N) are marked by black asterisks, the cysteine that forms the disulphide bridge between the subunits is marked by # and residues comprising the calcium ion binding site are underlined.



▶ **Fig. 3** **a** Schematic showing domain organisation of TPO: The protein sequence is coding an N-terminal signal peptide and propeptide domain (yellow), a MPO-like domain (green) with a catalytic heme (orange) that is connected to a CCP-like (Shamrock green), and an EGF-like (cyan blue) domain, an anchoring trans-membrane domain (dark blue) and an intracellular domain (dark magenta); **b** X-ray crystal structure of human myeloperoxidase (MPO) [43]. (PDB ID: 1CXP). MPO dimers are coloured by chain. Prosthetic heme group is shown as spheres [72]; **c** NMR solution structure of the Vaccina virus complement control protein (CCP) (PDB ID: 1VVD). Within the PDB, this structure most closely matches TPO's CCP-like domain (35 % identity); **d** NMR solution structure of a covalently linked pair of epidermal growth factor (EGF)-like domains from human fibrillin-1 [73]. (PDB ID: 1EMO). Within the PDB, this structure most closely resembles TPO's EGF-like domain (54 % identity). The EGF-like domain here contains 2 disulfide linkages that are shown as yellow sticks.

structed, facilitating mapping the regions of TPO that are responsible for its autoantigenicity [34]. The structural determination of closely related MPO [35] provided a much needed platform in order to predict the structural basis of TPO antigenicity. This prompted the design of TPO-MPO chimera proteins for mutagenesis studies in order to identify key residues that contribute to autoantigenic epitopes on TPO [36].

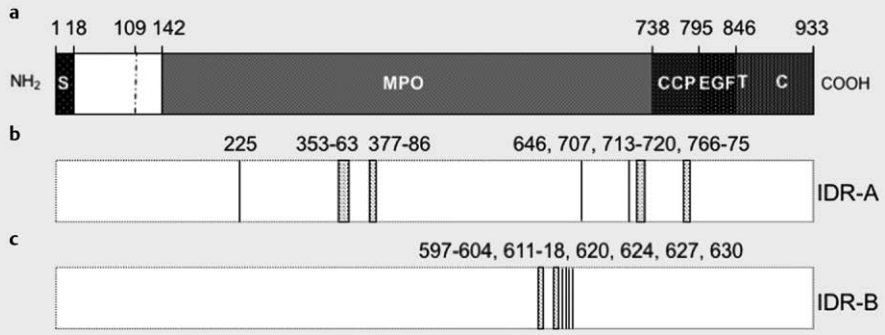
Over the last 20 years there have been considerable efforts in mapping the regions of TPO that may be responsible for its autoantigenicity using this homology approach. Several groups have shown that the immune response to TPO is directed towards two main surfaces on the protein termed immunodominant regions (IDRs) named A and B [34, 37, 38]. Autoantibodies that strongly interact with IDR-B are restricted to one major region (residues 597–630) of TPO whereas autoantibodies that bind IDR-A are scattered throughout the surface of the protein (▶ **Fig. 4**). The data suggest strongly that the majority of residues that compose these two epitopes are found on the MPO-like domain, but residues from the neighbouring CCP-like as well as the EGF-like domains are also involved [39, 40].

The first epitope mapping studies on TPO identified two immunodominant regions: IDR-A and IDR-B (▶ **Table 1**). Although initially defined with murine monoclonal antibodies [38, 41], they were independently identified using human anti-TPO antibody Fabs, which were also named A and B, but inverted in their ordering [37, 42]. IDR-A of the mouse mAbs corresponds to IDR-B of the human Fab fragments and vice versa. This review will refer to IDR-A and IDR-B as they were first defined by the mouse mAbs [38].

The Oligomeric State of TPO in Relation to its Autoantigenicity

Human LPO is the most closely related protein to TPO sharing 48 % sequence identity to the MPO-like domain of TPO. Crystal structures have been determined for human LPO and MPO [43, 44]; however, MPO is believed to be a more reasonable template for homology modelling as it forms disulfide-linked dimers, whereas LPO is monomeric [15]. Dimerisation of MPO occurs in the secretory pathway (post exit from the ER) during proteolytic processing. Notably, some (~ 10 %) of this MPO is secreted as a monomer and maintains full functionality [15]. The dimerisation of MPO does not appear to affect its activity and the functional consequences of dimerisation for MPO are not yet fully understood [15, 45]. There is considerable experimental evidence showing that native TPO exists as a membrane bound homodimer, linked via a conserved intermolecular disulphide bond at the same location as in MPO (TPO Cys296) at the dimer interface [19, 36, 46, 47]. TPO fusion proteins with FLAG and enhanced green fluorescent protein (eGFP) tags demonstrated TPO dimerisation when co-immunoprecipitation and enzyme-linked-immunosorbent assay (ELISA) binding studies were performed [47]. Interestingly, these homodimers were resistant to denaturation with conventional reducing agents. However, there are relatively few studies to explain the process of TPO dimerisation and where in the cell TPO dimers are formed.

Analysis of AITD patient serological activity in the context of structure and antigenicity of recombinant human TPO revealed higher recognition rates for TPO dimers over the reduced monomer [46]. More AITD patients were found to recognise the dimeric



▶ **Fig. 4** Schematic of the protein structure of full length TPO **a** and amino acid residues that constitute IDR-A **b** and IDR-B **c**. S: Signal peptide; MPO: MPO-Like domain; CCP: CCP-like domain; EGF: EGF-like domain; T: Transmembrane span; C: Cytoplasmic tail.

form of TPO over the monomer. This finding suggests that TPO dimers may contain antigenic determinants that are not present in the monomer.

It is generally accepted that human TPOAbs preferentially bind to native TPO [36, 48, 49]. Nevertheless, there is some proportion of autoantibodies, which are able to bind denaturated TPO [48, 50]. It is possible that TPO contains cryptic epitopes that are only revealed when the enzyme is unfolded or that dissociation of the dimer is likely to alter the conformational properties of TPO and thus its interaction with autoantibodies [51]. The possibility that there is antigenicity unique to both monomeric and dimeric forms of TPO is not excluded.

Given the complex nature of the autoantibody response to TPO and the highly complex post-translational processing involved in the assembly of the native enzyme, there may be unique antigenicity of both monomeric and dimeric forms of TPO. With many key studies relying on homology modelling with MPO without consideration of the spatial arrangement of epitopes, the oligomeric state and domain architecture of TPO in relation to its positioning in the membrane, much of the data gathered for TPO are unable to be placed within a structural context. Therefore, developing more advanced molecular models of TPO structure and the eventual determination of a crystal structure of TPO will be absolutely fundamental in providing insights into the molecular and structural determinants of TPO autoantigenicity. Not only will it provide an atomic positioning of the extensive epitope mapping data, but importantly, it will allow for the dissection of the interplay between TPO protein structure, function and autoantigenicity.

Structural Homology Modelling of Thyroid Peroxidase

TPO has been the target of structural studies for over 20 years. Low-resolution human TPO crystals were reported over 15 years ago [52, 53], however, to date, no high-resolution full-length mammalian TPO structure has been determined and the structural determinants of autoantigenicity still remain unknown. In this time, there have been considerable efforts in mapping the epitopes of monoclonal autoantibodies onto the TPO molecule. This has allowed the low resolution identification of regions of TPO that are recognised by antibodies. However, the tertiary and quaternary structural context of TPO immune recognition has not been firmly established.

Development of advanced molecular models of TPO are critical for the interpretation of a large body of epitope mapping data in the absence of a crystal structure. This information can be used to determine the structural basis for interaction between TPO and its autoantibodies. In addition, molecular models of TPO will provide functional insight into how it binds its two substrates, iodide and thyroglobulin. It will also shed light on the nature of the iodinating species and the mechanisms involved in their binding to tyrosine sites on thyroglobulin during the process of thyroid hormone biosynthesis.

Modelling The Structure and Architecture of the Extracellular Domains of TPO

High sequence identity between human TPO and proteins with known structures has enabled homology modelling of the three extracellular domains [34, 51, 54, 55]. A crystal structure is available for MPO, which like TPO functions as disulfide-linked dimer [34, 46, 47] Therefore, the MPO-like domain (residues 142–738) could be modelled using the crystal structure of human MPO. The dimer interface in the MPO structure defines the relative orientation of the monomers in the TPO model. Unlike other known peroxidases, TPO has a transmembrane (TM) domain, as well as CCP-like and EGF-like domains linking the MPO-like domain and the TM domain (▶ **Fig. 3a**). The CCP-like (residues 739–795) and EGF-like (residues 796–846) domains likewise have high sequence identity homologues for which structures are known (▶ **Fig. 3d** and ▶ **Fig. 5**). Together, this has enabled homology modelling of all three extracellular domains of TPO [51].

Transmembrane Helix Association

A 25 amino acid sequence has previously been identified as a likely transmembrane domain using hydropathy profiling (amino acid sequence WISMSLAALL**IG**GFA**G**LTS**T**VICRW, residues 849–873)

▶ **Table 1** The identities of epitopes and residues on the TPO molecule as defined in various studies constituting two immunodominant regions A and B involved in antibody binding.

Epitope Residues	Length	Study	Antibodies involved
IDR-A			
P377–R386	10	Phage display technology to identify critical motifs involved in the binding of human autoantibodies [41]	T13
H353–Y363	11	Combination of site-directed mutagenesis, flow cytometry and ELISA to identify key residues involved in the antigen-antibody interaction [41–42]	T13, ICA1
K713–S720	8	Directed mutagenesis and antibody binding kinetic studies by surface plasmon resonance to further confirm interaction [43]	T13, TR1.9
K713	1	Rational mutagenesis studies based on a predicted structural model for TPO [44]	TR1.9
D707	1	Rational mutagenesis studies based on a predicted structural model for TPO [45]	126TO10, 126TP1
R225	1	Recombinant human Fabs from autoimmune patients and TPO mutants to identify key residues involved in binding [46]	126TP1, 126TO10, 126TP7
R646	1	Rational mutagenesis studies based on a predicted structural model for TPO [45]	126TO10, 126TP1
Y766–Q775	10	Phage display technology to identify critical motifs involved in the binding of human autoantibodies [41,47]	T13
IDR-B			
D630	1	Rational mutagenesis studies based on a predicted structural model for TPO [45]	126TP14, 126TP5
K627	1	Recombinant human Fabs from autoimmune patients and TPO mutants to identify key residues involved in binding [46]	126TP14, 126TP5, 131TP7
D624	1	Rational mutagenesis studies based on a predicted structural model for TPO [45]	126TP14, 126TP5
D620	1	Rational mutagenesis studies based on a predicted structural model for TPO [45]	126TP14, 126TP5
T611–V618	8	Competitive studies using all available reference anti-TPO antibodies to further define the IDR [48]	TR1.8
F597–E604	8	Competitive studies using all available reference anti-TPO antibodies to further define the IDR [48]	126TP14, 126TP5, SP1.4, WR1.7

[56]. Bioinformatic analysis of this region predicts an α-helical conformation, and a transmembrane helix dimerisation motif, G^{860}xxxG^{864}[51]. The GxxxG motif is indicative of a dimeric coiled-coil structure of transmembrane helices [57–59]. There are two additional features that support transmembrane helix dimerisation in TPO. First, the isoleucine that precedes the first glycine in the motif is frequently seen in transmembrane helices [59]. Second, there is a threonine in the 4th position after the GxxxG motif (▶ **Fig. 2**). GxxxGxxxT is the most represented three-residue pattern subset of the LIxxGVxxGVxxT seven-residue helix-helix dimerisation motif [59, 60]. The incorporation of this predicted transmembrane helix dimerisation motif can therefore be modelled into the architecture of TPO with a high degree of confidence.

TPO Domain Organisation And Enzyme Function

Although the MPO-like domain of TPO can be modelled as a dimer using the known crystallographic disulfide-linked dimer of MPO, the spatial organisation of the MPO-like domains relative to the CCP-like and EGF-like domains as well as the membrane cannot be determined from primary sequence alone. There are several factors that allow for two alternative arrangements: the order of the domains in the TPO primary sequence, symmetry restraints, the dimerisation interface inferred from the structure of MPO, and the predicted dimerisation in the TM domain. Modelling revealed that two alternative dimeric arrangements exist, which differ in their orientation of the MPO-like domain (▶ **Fig. 6**). Both orientations are both equally plausible from a purely structural perspective.

To determine which of these two arrangements is the most likely architecture, consideration of the enzymatic function of TPO must be taken into account. TPO catalyses two vital reactions in the biosynthesis of thyroid hormones: iodination of tyrosine residues in thyroglobulin, and coupling of the resulting iodotyrosines to form thyroid hormones T_3 and T_4[1]. This process is dependent upon the heme group being covalently bound to TPO [61]. Thus, the two possible models for TPO orient the cavity containing the heme group facing either the thyroid follicular lumen (the “trans” model; ▶ **Fig. 6a, c**) or the thyrocyte membrane (the “cis” model; ▶ **Fig. 6b, d**). Since iodination of thyroglobulin is thought to occur without a direct interaction between TPO and Tg, both models are equally plausible from an enzymatic standpoint [62].

The Plausibility of the Cis and Trans Models of TPO in Terms of Enzymatic Function

The trans model places the heme group necessary for TPO activity facing toward the follicular lumen, whilst the cis model places the heme pocket facing towards the cell membrane. Either model

▶ **Fig. 5** Sequence alignment of template structures to TPO sequence, allowing the construction of a TPO homology model. Immunodominant epitopes are boxed and labelled. Domains and motifs are indicated by colored bars.

would not prevent TPO accessing its substrates of iodide and hydrogen peroxide. However, the cis model would preclude the access of large proteins such as thyroglobulin (a 660 kDa homodimer), which provides tyrosine residues for iodination by TPO, due to the constricted space between TPO and the thyrocyte membrane. It is not known if Tg is iodinated by binding to TPO and forming an intermediate Tg/TPO complex. Thus both the cis and trans models of TPO are both valid in terms of what is known about TPO's enzymatic function.

Antibody Epitope Recognition in the Context of TPO Models

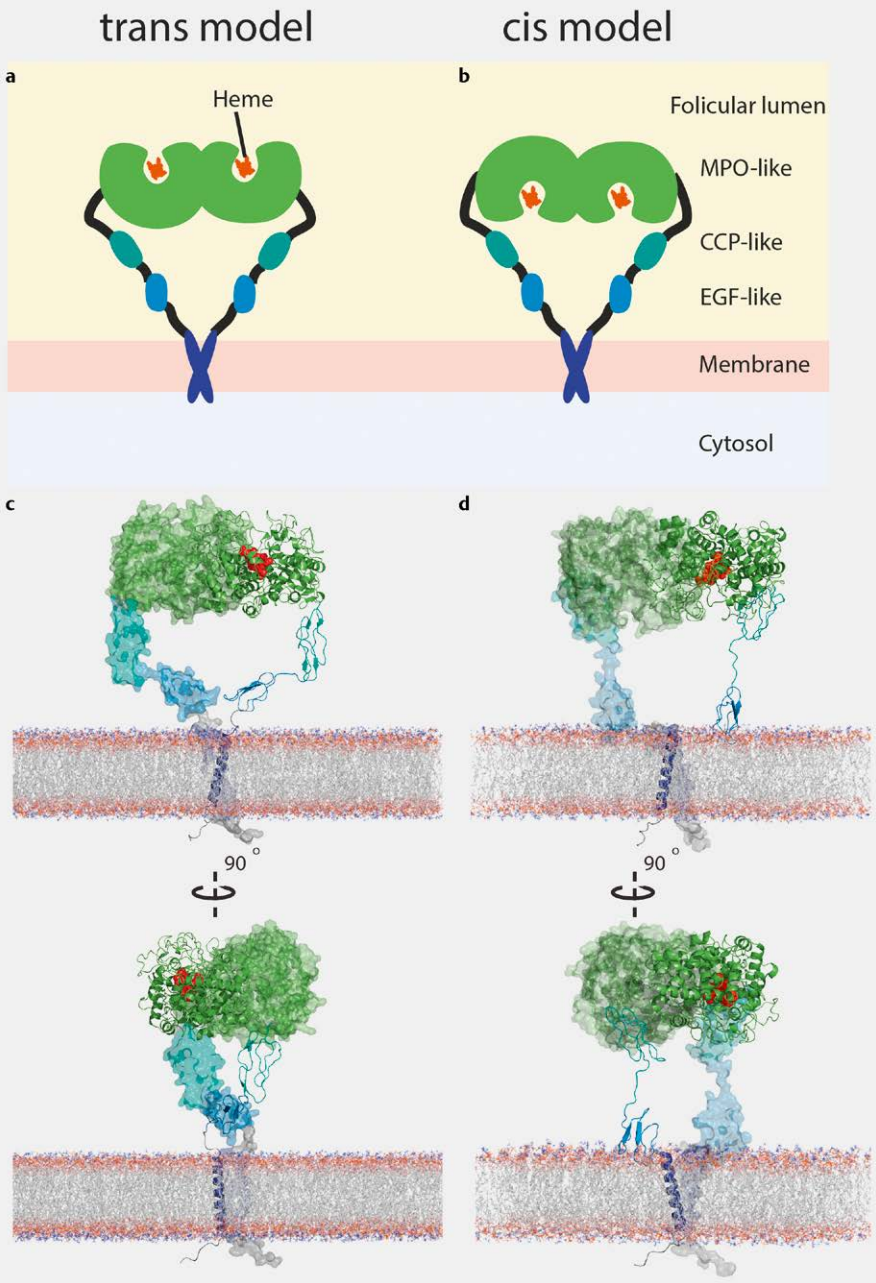
The extensive epitope mapping data obtained for TPO has thus far been unable to be visualised and interpreted within a 3D structural context that includes all TPO domains, interactions with the membrane and dimerisation. Mapping of these epitopes to which autoantibodies bind onto both trans and cis TPO models discussed above reveals that many of the residues identified that comprise the IDRs (from ▶ **Table 1**) recognise conformational determinants that are exposed on the enzyme surface (▶ **Fig. 7** and ▶ **Fig. 8**). From our models, it is clear that the MPO-like domain constitutes the main immunodominant region (IDR) to which autoantibodies react. Moreover, in both trans and cis TPO models, IDR-B closely arranges in a cluster near the MPO-MPO dimer interface whereas IDR-A is scattered across the surface of the MPO-like domain with one epitope on the CCP-like domain.

IDRs Mapped onto the Trans Model

Mapping IDRs onto the trans model of the TPO dimer places IDR-B on the thyrocyte membrane facing side of the MPO-like domain, in a closely arranged cluster near the MPO-MPO dimer interface whereas IDR-A faces the thyroid follicular lumen and is solvent accessible (▶ **Fig. 7a**). Considering the availability of epitopes to antibodies, IDR-B appears to be somewhat occluded in comparison to IDR-A (▶ **Fig. 7a**). The clustering of IDR-B on the trans model suggests that these epitopes constitute a major region on TPO's surface to which autoantibodies react and is an important feature that can be visualised on our models.

IDRs Mapped onto the Cis Model

Mapping immunodominant regions onto the cis model of the TPO dimer shows that IDR-B clusters closely together near the dimer interface on the lumen facing side of the TPO molecule and is fully solvent-accessible (▶ **Fig. 8a**). IDR-A is surface exposed and scattered across the molecule, as in the trans model, but in much closer proximity (▶ **Fig. 8a**). Given TPO's likely orientation in the

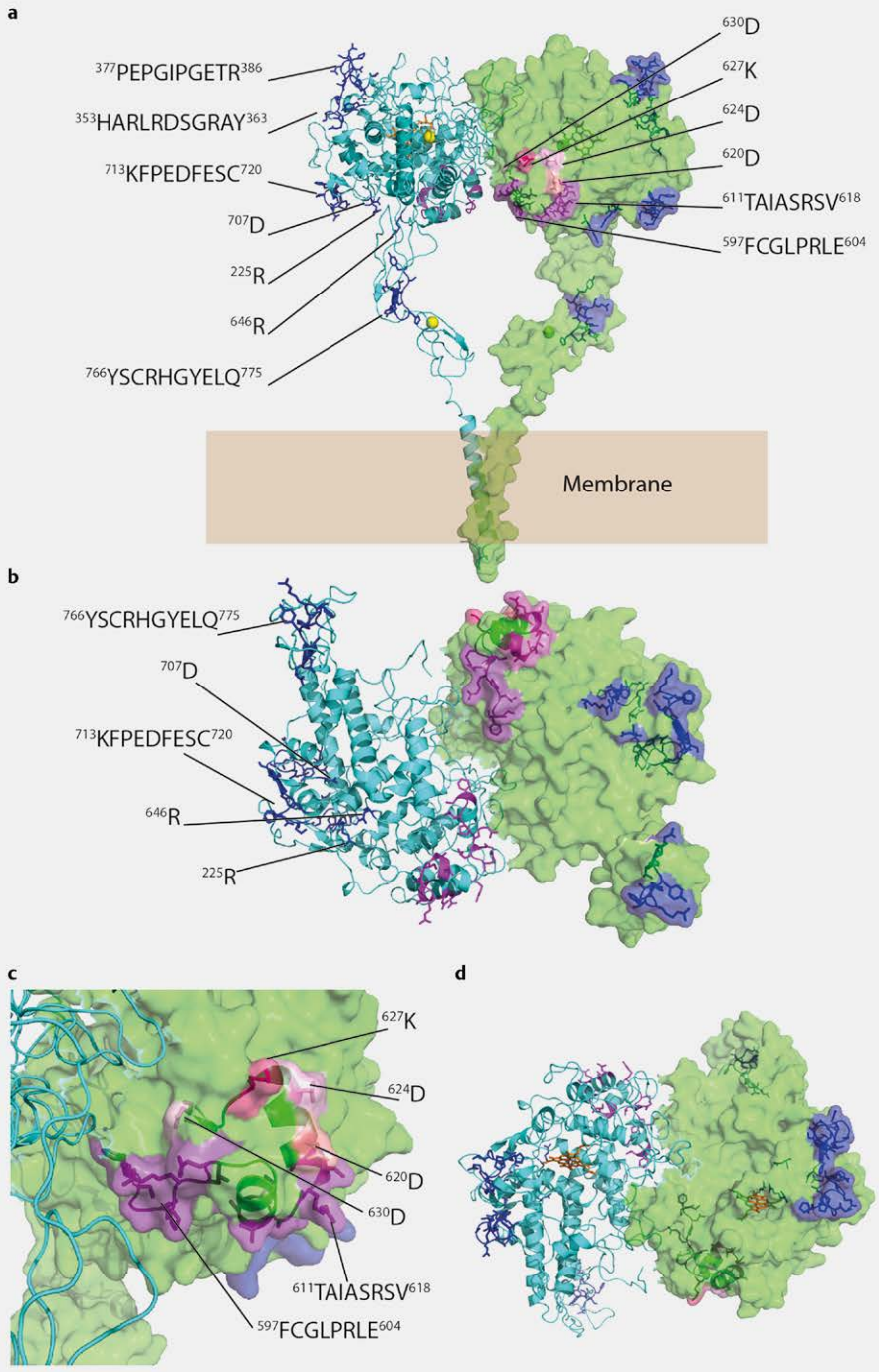


▶ **Fig. 6** A comparison between membrane-embedded, dimeric models of TPO isoform1 in two different conformations. **a** Schematic of trans model with active site facing away from follicular membrane. Colouring as in ▶ **Fig. 3**; **b** Cis model with active site facing towards follicular membrane; **c** perpendicular views of trans model; **d** perpendicular views of cis model. TPO represented as cartoon and space filling (subunits A and B, respectively) for clarity. Domains are coloured as follows: MPO-like domain (green), CCP-like domain (Shamrock green), EGF-like domain (cyan-blue), and TM domain (dark-blue). Catalytic heme represented as red spheres, and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) molecules represented as lines and coloured as spheres.

membrane, the epitopes mapped onto the cis model face the lumen and may be more easily accessible to autoantibodies. In both instances, IDR-B mapped onto both membrane-bound orientations of our TPO models shows that this region of the molecule plays a major role in TPO autoantibody reactivity and autoantigenicity.

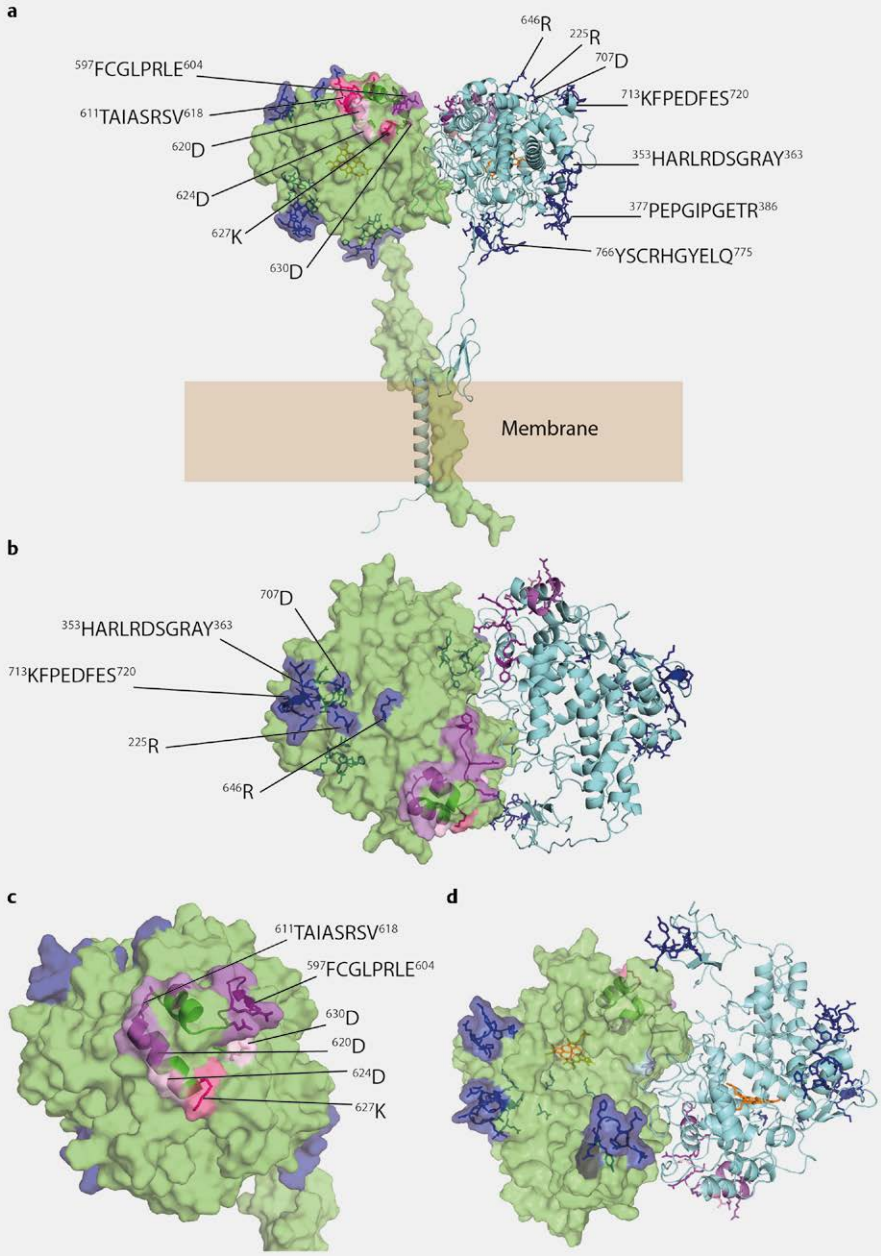
IDR-A is Widely Separated on Both Models of TPO

Taken together, the epitopes of IDR-A, mapped onto the trans and cis model are not as closely grouped together as the epitopes of IDR-B (▶ **Fig. 7a** and ▶ **Fig. 8a**). In both the trans and cis models,



▶ **Fig. 7** Mapping epitopes onto the trans TPO dimer. **a** Epitopes and residues identified in various studies as constituting immunodominant regions IDR-A (blue) and IDR-B (pink); **b** Membrane-facing side of MPO-like domain; **c** Close-up shows that much of the two immunodominant regions are on the membrane-facing surface of the MPO-like domain, particularly IDR-B; **d** Lumen-facing side of TPO. Heme groups are shown in orange.

these epitopes are spread across either side of the entire MPO-like domain (90 Å across the trans model and 80 Å across the cis model). These distances are too great to be simultaneously engaged by the CDR of an antibody. In each case, the epitopes on the CCP-like domain (residues 766–775) are too distant from the MPO-epitopes of IDR-A. These epitope mapping results thus invites a review into the studies that defined this region. Alternatively, the existing epitope mapping data may be interpreted with molecular TPO models in which conformational changes bring together the epitope regions on the CCP-like domain as such that it coalesces into one compact epitope along with the epitopes on the MPO-like domain, as shown for IDR-B in the homology models discussed.

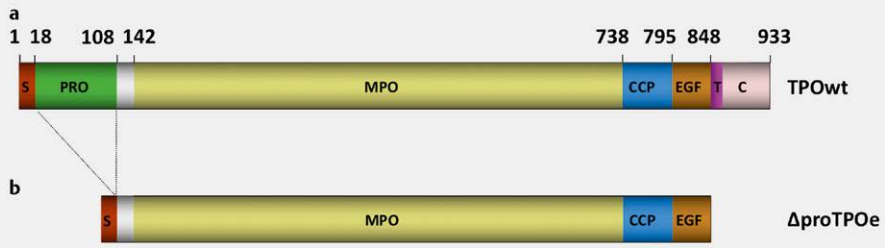


▶ **Fig. 8** Mapping epitopes onto the cis TPO dimer. **a** Epitopes and residues identified in various studies as constituting immunodominant regions IDR-A (blue) and IDR-B (pink); **b** Lumen-facing side of MPO-like domain; **c** Close-up shows that much of the two immunodominant regions are on the lumen-facing surface of the MPO-like domain near the dimer interface, particularly IDR-B; **d** Membrane-facing side of TPO containing the heme (orange) cavity.

Oligomeric Architecture of TPO Plays a Role in its Autoantigenicity

The findings of epitope mapping data of IDR-B suggests that engagement of antibodies with IDR-B on both trans and cis models of TPO may require a conformational change of TPO, and perhaps even dissociation of the dimer into monomers. In the case of IDR-B in the trans model, some residues that constitute the epitopes are buried and not surface exposed (▶ **Fig. 7a**). Again, in the case of the IDR-B in the cis model, the dimer interface may partially block full antibody engagement as some residues of the epitopes are buried by the dimer interface (▶ **Fig. 8a**). TPO has been identified in both monomeric and dimeric–forms and may be presented to the immune response as a monomer TPO [46, 63–66].

The epitopes to which antibodies recognise provide clues to the structure of the original antigen. Interestingly, mapping the epitopes onto models shows that dimerisation does not bring together IDRs from respective monomers in either cis or trans models (▶ **Fig. 7a** and ▶ **Fig. 8a**). IDR-B, which is located on the under-



▶ **Fig. 9** Schematic showing engineered TPO constructs. **a** Full length human TPO; **b** TPO ectodomain lacking the propeptide. S: Signal peptide; MPO: MPO-Like domain; CCP: CCP-like domain; EGF: EGF-like domain; T: transmembrane span; C: Cytoplasmic tail.

side of the MPO-like domain near the MPO-MPO interface on the trans model (▶ **Fig. 7b**), shows the closest proximity of epitopes from each monomer, but they are still too separated to participate in the same antibody-binding interaction in a single epitope. While the model supports a TPO dimer within the membrane, it is plausible that the original antigen could have been in a monomeric arrangement.

Taken together, the modelling suggests that the cis dimer is slightly more favourable over the trans dimer, based upon the spatial arrangement and accessibility of epitopes. It also suggests that epitopes may be partially hidden during dimerisation. Both cis and trans architectures may implicate significant conformational plasticity upon engagement with autoantibodies, suggesting that the oligomeric state and conformational properties of TPO play important roles in its autoantigenicity. These findings invite further investigation of the relationship between antibody binding and TPO's oligomeric state.

Engineering and Design of Human TPO Constructs

During maturation TPO is gradually trimmed at the N-terminal region. The cleavage sites for endoproteases, which are responsible for this process, are located in the propeptide sequence [67]. The process of endoproteolysis is at least partly responsible for low homogeneity of TPO protein purified for structural studies [11]. In an attempt to improve TPO preparation quality, a TPO cDNA lacking propeptide sequence was engineered and stably expressed in the Chinese Hamster Ovary (CHO) cell line [19]. Complex analyses conformed that propeptide-deprived TPO exhibits similar functional and immunogenic properties as full-length TPO. Importantly, truncated TPO protein is insensitive to intramolecular proteolysis [19]. In further studies we engineered other TPO cDNA constructs (▶ **Fig. 9b, c**), which can be used to obtain significant quantities of soluble TPO of high biochemical and antigenic quality in mammalian cell-based protein expression systems (Godlewska, Gora, Buckle and Banga, unpublished data). We have further attempted to stabilise a TPO dimer by adding a leucine zipper dimerisation domain to the C-terminus. Data generated by us using this construct has shown that recombinant TPO exists in both forms, and that a patient-derived autoantibody Fab (TR1.9) can preferentially bind the monomer (Williams, Godlewska, Hoke and Buckle, unpublished).

Conclusions/Future Directions

Without the presence of an experimentally determined atomic structure and appreciation of its structure in a physiological membrane-bound form, it remains difficult to precisely delineate the nature and structural basis of TPO antigenicity. Furthermore, there are relatively few structures reported for autoantigen/Fab complexes: the TSH receptor in complex with a thyroid-stimulating [68] and blocking autoantibody [69], the MOG-8-18C5-Fab complex [70], and IgG Fc-IgM-RF-Fab complex [71]. Therefore, little information in regards to structural insights and common features of autoantigen-autoantibody interactions can be elucidated.

However, a molecular modelling approach has advanced the visualisation and interpretation of epitope mapping studies for TPO, providing a much needed platform for structurally interpreting epitope data and highlighting new avenues for investigation of the breakdown of immune tolerance to TPO in thyroid autoimmune disease. Both trans and cis models of dimeric TPO are equally likely based upon conformational properties as well as enzyme function, with the cis model being slightly more favourable based upon spatial positioning of epitopes. Visualisation of the extensive epitope mapping data on both models of the TPO dimer provides structural insights into the basis of TPO autoantigenicity and a platform for further investigation. Modelling has also produced testable TPO constructs that are enzymatically active and immunologically relevant, and has shown that the MPO-like domain of TPO constitutes the main immunodominant region to which autoantibodies react. Moreover, engineered constructs lacking the propeptide, TM domain and cytoplasmic tail, do not affect TR1.9 Fab autoantibody binding to conformational epitopes. These proteins can be used as tools to test the role of TPO dimerisation in autoantibody recognition and eventual X-ray crystallography structural determination of TPO alone and in complex with autoantibodies. It is envisaged that further structural and functional characterisation of TPO will provide significant advances. For example, oligomerisation can be investigated using Analytical Ultra Centrifugation (AUC), and shape inferred using small angle X-ray scattering (SAXS). Autoantibody binding to TPO constructs and characterisation of binding affinities can be achieved by Surface Plasmon Resonance (SPR/Biacore). Limited proteolysis may provide structural information on TPO domain architecture and flexibility of TPO ectodomains, perhaps also yielding more suitable and stable proteins for structural determination. Ultimately, 3D structural determination of TPO alone and in complex with autoantibodies by X-ray crystallography or Cryo electron

microscopy will achieve the ultimate aim of providing atomic level detail and molecular insights in regards to TPO interactions and autoantigenicity.

Contribution Statement

DEW, SNL, MG, DEH, and AMB wrote the paper.

Conflict of Interest

The authors declare that they have no conflict of interest.

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