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# Application of peptide antibodies to studies on the immunodominant conformation dependent epitopes of human thyroid peroxidase**

# Zastosowanie przeciwciał peptydowych w badaniach nad konformacyjnymi epitopami immunodominującymi w ludzkiej peroksydazie tarczycowej

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S u m m a r y

The discontinuous immunodominant regions (IDRs) recognized by autoantibodies directed to thyroid peroxidase (TPO) have not been unequivocally localized. We have explored the location of the IDRs by generation rabbit anti-TPO peptide antibodies and competition experiments with monoclonal antibodies (moabs) specific for those IDRs. Previously we suggested the localization of IDR-A and IDR-B and here we tested the validity of our conclusions. The specificity, reactivity with native TPO, cross reactivity with homologous proteins, the effect of steric hindrance, and the possibility of conformational changes induced in TPO by peptide antibody binding have been explored. The inhibition of IDR-B specific moabs and autoantibodies binding to TPO approaching 90% by peptide antibodies or their mixture call for an explanation and we think that at least part of those peptide amino acid sequences could be involved in building the IDR-A and IDR-B regions.

Key words: thyroid peroxidase, autoantibody epitopes, peptide antibodies, immunodominant regions

S t r e s z c z e n i e

Lokalizacja nieciągłych regionów immunodominujących (IDR) rozpoznawanych przez autoprzeciwciała skierowane przeciwko peroksydazie tarczycowej (TPO) nie została w pełni poznana. W prezentowanej pracy zbadaliśmy lokalizację nieciągłych regionów immunodominujących (IDR) poprzez wytworzenie króliczych przeciwciał skierowanych przeciwko peptydom TPO oraz kompetycyjne eksperymenty z monoklonalnymi przeciwciałami (moabs) specyficznie wiążącymi się z regionami IDR. Sprawdziliśmy, czy potwierdzi się wcześniej przez nas zaproponowana lokalizacja regionów IDR-A i IDR-B. Określiliśmy specyficzność, reaktywność z natywną TPO, reaktywność krzyżową z homologicznymi białkami, efekt zawady sterycznej i potencjalną możliwość zaistnienia zmian w konformacji TPO indukowanych przyłączaniem się przeciwciał specyficznie wiążących peptydy. Inhibicja wiązania z TPO monoklonalnych przeciwciał specyficznych dla IDR-B i autoprzeciwciał przez pojedyncze przeciwciała anty-peptydowe lub ich mieszaniny osiągała poziom 90%. To pozwala sądzić, że przynajmniej część badanych sekwencji aminokwasowych peptydów wchodzi w skład struktury regionów IDR-A i IDR-B.

Słowa kluczowe: peroksydaza tarczycowa, epitopy dla autoprzeciwciała, przeciwciała peptydowe, regiony immunodominujące

## INTRODUCTION

Thyroid peroxidase (TPO) is responsible for the thyroid hormone biosynthesis (1). TPO is also one of the autoantigens in disorders such as Hashimoto's thyroiditis and Graves' disease, which are the most common human autoimmune diseases (2, 3). Autoantibodies to TPO are polyclonal and recognise discontinuous immunodominant regions (IDRs) on the molecule (4, 5). The major part of the autoantibody response to TPO is directed towards two regions, which were defined with a panel of murine monoclonal antibodies (moabs), termed IDR-A and -B (4). These IDR regions of TPO have not been unequivocally identified so far. Several techniques have been used in elucidating the location of IDR on the TPO molecule. Chimeric molecules of TPO-myeloperoxidase (6, 7) allowed exclusion of two

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major areas of TPO from involvement in IDRs of TPO. Studies on large recombinant fragments of TPO suggested the involvement of amino acids 590-622 and 709-721 (8). Deletion of large segment of TPO suggested the importance of sequence 386-652 (9). Studies on proteolytic fragments of TPO suggested an involvement of C-terminal amino acids (742-848) in building up the IDRs (10, 11). This finding have been questioned by others (12) as the recombinant TPO with truncated C-terminal (1-741) was precipitated by four monoclonal antibodies as well as TPO 1-771 and the full TPO ectodomain. The participation of the EGF-like domain (796-841) has been excluded as being a part of the IDR (13), while some evidences has been presented for the involvement of the CCP-like domain (739-795). Footprinting experiments suggested the participation of Lys 713, but it location at the fringe of IDR is possible (14). Other studies with recombinant TPO fragments suggested the involvement of a junction region between the MPO- and CCP-like domain of TPO (13, 15). An earlier work using recombinant TPO fragments (16) suggested the involvement of amino acid sequence 513-633 in building up the IDR, the importance of the sequence 589-633 was underlined. We have described that anti-TPO peptide antibodies to part of the sequence (599-617) described by Arscott et al. (16) strongly inhibit autoantibodies and IDR-B specific moabs binding to TPO (17). Using the model of TPO and the known localization of one of the IDR-A specific moab 47, we obtained antibodies to peptides covering the whole surface between and around the moab 47 and the sequence 599-617 (18), and found that a mixture of rabbit antibodies to this region inhibit binding of IDR-A and -B specific recombinant fab fragments and autoantibodies to TPO up to 90%. Bresson and co-workers (19) in an elegant paper described that four regions are taking part in building up the IDR for one human monoclonal antibody to TPO (moab T13), by replacement of 8 to 10 amino acids sequence. These results are in apparent conflict with our results as they do not involve the sequences described by us (17, 18). Strong inhibition of autoantibodies, moabs, and recombinant fabs binding to TPO by rabbit peptide antibodies are clear cut, however, interpretation of these results might be more complicated and we have addressed these questions in the present work.

## MATERIALS AND METHODS

### Synthesis of peptides and modeling of TPO structure

The molecular model of TPO, based upon the homologous structure of MPO, has been described previously (17). All the synthetic peptide sequences used in this study (tab. 1) correspond to sequences in the MPO- and CCP-like domain of TPO. The location and solvent accessibility of some of these peptides such as P6, P14, P15, P16, and P17 has been described (17). The other peptides were selected by inspection of the model to cover the TPO surface around and between the epitope for moab 47 (713-721) and our peptide P14 (599-617). All peptides were synthesized by F-moc chemistry with C-terminal amides and a cysteine residue at the N- or C-terminus for coupling to carrier protein as described earlier (17). All peptides were checked for purity by mass spectrometry.

### Antibodies

Mouse moabs to TPO were obtained from Dr. J. Ruf (4). Serum from patients with thyroid autoimmune disease was obtained from the Warsaw Outpatient Endocrine Clinic. Pooled serum from normal healthy individuals (n = 20) was used as a control. Pooled sera from 20 patients with thyroid autoimmune disease, positive for TPO antibodies, were used as positive control for experiments with human sera. Autoantibodies to TPO were measured by ELISA, standardized to the WHO/ /MRC international standard 66/387 (17). Peptides were conjugated to maleimide activated keyhole limpet hemocyanin (KLH) (1 mg peptide/1 mg KLH) and further purified by chromatography on Sephadex G-100 in PBS (17). At least two New Zealand White rabbits per peptide were injected according to the described schedule (17). All antisera were tested for reactivity to human proteins (albumin, IgG, thyroglobulin), bovine albumin, and egg albumin. All antisera were also tested for reactivity with human myeloperoxidase and lactoperoxidase.

All experiments with animals were approved by the Warsaw Ethical Committee for Experiments on Animals no. 55/01.

### Purification of hTPO

Human TPO was prepared from pooled Graves' thyroid tissue as described (20). TPO preparations used for ELISA were further purified by affinity chromatography on protein L-Sepharose. A column containing 2 ml of protein L-Sepharose (Actigen) was washed with 20ml of Tris-buffered saline (TBS) pH 8.0 containing 0.05% deoxycholate (DOC) followed by TPO solubilisation in the same buffer. The column was incubated for 1 h at room temperature, washed with TBS containing 0.05% DOC and the non-retained fraction collected and concentrated for use in the ELISA experiments. This step removes almost all IgG contamination from the TPO preparation.

### ELISA and inhibition of antibodies binding to TPO

All ELISA tests were performed as described previously (17). In short, microtitre plates (Nunc) were coated with purified human TPO (or other protein at 1 $\mu$g/ml), 100 $\mu$l of diluted rabbit anti-peptide serum added and incubated for 1 h at room temperature. After washing three times with PBST, HRP-conjugated goat anti-rabbit autoantibodies were added to the wells and incubated for 1 h at room temperature. The plates were developed with TMB solution and the optical density (OD) was measured at 450 nm.

Inhibition or enhancement of autoantibodies' and moab's binding to TPO was performed on TPO coated

Table 1. Rabbit anti-peptide antibodies.

| Peptide number | TPO sequence | Titer with peptide | Titer with TPO | Titer with MPO* | Titer with LPO* | Inhibition by native TPO** (%) |
|---|---|---|---|---|---|---|
| P1 | 225-242-C | 102.400 | 51.200 | 400 | 1.600 (60%) | 8 |
| P2 | 403-421-C | 12.800 | 2.000 | 400 | 800 | 16 |
| P4 | 451-469-C | 12.800 | 51.200 | 0 | 0 | 42 |
| P5 | 489-507-C | 6.400 | 8.000 | 0 | 0 | 0 |
| P6 | 503-516-C | 25.400 | 8.000 | 0 | 0 | 0 |
| P8 | 618-636-C | 12.800 | 32.000 | 0 | 0 | 0 |
| P9 | 662-680-C | 12.800 | 2.000 | 400 | 0 | 0 |
| P10 | 679-696-C | 3.200 | 2.000 | 0 | 0 | 8 |
| P11 | 574-587-C | 256.000 | 128.000 | 0 | 16.000 (80%) | 48 |
| P12 | 549-563-C | 64.000 | 256.000 | 800 | 0 | 63 |
| P13 | 567-581-C | 128.000 | 128.000 | 0 | 0 | 45 |
| P14 | 599-617-C | 256.000 | 256.000 | 0 | 200 | 95 |
| P15 | 610-622-C | 128.000 | 32.000 | 0 | 0 | 20 |
| P16 | 189-201-C | 320.000 | 320.000 | 400 | 400 | 36 |
| P17 | 179-190-C | 640.000 | 40.000 | 200 | 200 | 38 |
| P18 | C-210-225 | 64.000 | 32.000 | 800 | 800 | 0 |
| P19 | 468-477-C | 256.000 | 64.000 | 0 | 0 | 0 |
| P20 | C-721-728 | 256.000 | 1000 | 0 | 0 | 46 |
| P21 | 249-256-C | 128.000 | 64.000 | 0 | 400 | 23 |
| P22 | 536-546-C | 256.000 | 32.000 | 3.200 (60%) | 16.000 (88%) | 17 |
| P23 | C-425-438 | 128.000 | 1.600 | 0 | 0 | 10 |
| P24 | C-375-387 | 320.000 | 320.000 | 0 | 0 | 61 |
| P25 | C-389-398 | 640.000 | 16.000 | 0 | 0 | 0 |
| P26 | 607-617-C | 128.000 | 64.000 | 0 | 0 | 0 |
| P27 | C-697-710 | 256.000 | 640.000 | 100 | 1.600 (40%) | 38 |
| P28 | 222-229-C | 32.000 | 64.000 | 0 | 200 | 0 |
| P31 | C-461-476 | 256.000 | 128.000 | 200 | 200 | 52 |
| P32 | 273-286-C | 256.000 | 64.000 | 0 | 0 | 22 |
| P35 | 642-650-C | 64.000 | 100 | 0 | 0 | 68 |
| P37 | C-756-767 | 64.000 | 8.000 | 0 | 400 | 0 |
| P39 | C-604-612 | 256.000 | 1.024.000 | 0 | 0 | 68 |
| P41 | C-742-755 | 128.000 | 16.000 | 0 | 0 | 25 |
| P42 | 599-607-C | 128.000 | 6400 | 0 | 0 | 15 |
| P43 | 702-721-C | 256.000 | 256.000 | 0 | 200 | 73 |
| P46 | 603-617-C | 32.000 | 16.000 | 0 | 0 | 45 |
| P50 | 353-372-C | 256.000 | 16.000 | 0 | 200 | 27 |
| P51 | 321-340-C | 256.000 | 64.000 | 3.200 (70%) | 16.000 (81%) | 51 |
| P53 | 287-296-C | 128.000 | 2.000 | 0 | 400 | 21 |
| P54 | 760-779 | 128.000 | 128.000 | 0 | 200 | 56 |
| P55 | 599-611-C | 64.000 | 64.000 | 0 | 0 | 42 |
| P56 | 599-609-C | 64.000 | 64.000 | 0 | 0 | 32 |

*Percent of homology with myeloperoxidase (MPO) or lactoperoxidase (LPO) in brackets.
**Inhibition of antibodies binding to TPO coated on polystyrene plates by the same TPO preparation in solution.

plates incubated for 18 h at 10°C with 100x diluted rabbit anti-peptide serum. After washing, patients' sera, which had been diluted to give 5 IU/ml of TPO autoantibodies, were added to the wells and incubated for 1 h at room temperature. Experiments with moab's were carried out at moab concentration of 50% of maximal binding. After washing three times with PBST, HRP-conjugated rabbit anti-human IgG (diluted 1:2000) or

rabbit anti-mouse was added and incubated for 1 h followed by three washes with PBST. The plates were developed with TMB solution and the optical density was read at 450 nm. Pre-immune rabbit serum was used for controls and wells without addition of human serum were considered as blank. HRP conjugated antibodies were obtained from Sigma-Aldrich.

Inhibition or enhancement of an antibody binding was calculated in comparison to pre-immune serum according to the formula:
A-B/T-B x 100,
where B = OD background, T = OD pre-immune serum, and A = OD anti-peptide serum.

## RESULTS

Rabbit antibodies to forty one peptides of the MPO- and CCP-like domain of human thyroid peroxidase have been obtained (tab. 1). The anti-TPO peptides antibodies react at high titter with the peptide used for immunization, majority of them react well with TPO in ELISA assay and some of them react also with homologous protein, that is lacto- and myeloperoxidase (tab. 1). All of the peptide antibodies reacted well with denatured TPO after SDS electrophoresis in blotting experiments (results not shown). The binding of antibodies to the native protein could be assessed by inhibition of antibodies binding to TPO coated on polystyrene plates by native protein (21). Ten peptide antibodies did not react with native TPO (0% inhibition), seven showed weak reaction (8-20% inhibition), fifteen had moderate activity (21-50% inhibition) and nine binded well to the native TPO (51-95% inhibition) (tab. 1). The cross reactivity of peptide antibodies with other proteins has been tested with human thyroglobulin, human albumin, human IgG, bovine albumin, and egg albumin. Only antiserum to P14 reacts with human thyroglobulin at a titer 1:4000. Anti-P4 reacted with human IgG at a titer below 1:1000, and anti-P4, -P5, -P9, -P27, and -P31 reacted with bovine albumin at titers below 1:1000. All of the rest peptide antibodies did not react with human thyroglobulin, human albumin, human IgG, bovine albumin, and egg albumin (results not shown).

We have demonstrated previously (17) that antibodies to peptide P14 could inhibit the binding of autoantibodies and IDR-specific moabs to TPO. This was explained as cross reactivity of anti-P14 antibodies with the IDR-B region on TPO (17). To further study this phenomenon we obtained anti-P14 antibodies in six rabbits and antibodies to six fragments of P14 (peptide P26, P39, P42, P46, P55, and P56).

Antisera from rabbit no. 1 to 4 inhibited binding of IDR-B-specific monoclonal antibodies (moab 15, 16, and 64) by 90 or more percent (tab. 2). Also sera from those four rabbits inhibited the binding of human autoantibodies to TPO by 39-42% (tab. 2). Sera from rabbits no. 5 and 6 did not inhibit the binding of human autoantibodies to TPO and only moab 15 binding was inhibited to a significant extent. The binding of control moabs (moab 2 and A4) to TPO was not significantly influenced by any of those six rabbit sera. Moab 2 reacts with the IDR-A region (4) and moab A4 (22) with a linear sequence located in the region of TPO that does not belong to IDR-A and -B (unpublished data). All of the peptide antibodies to the 6 fragments of P14 (peptide 26, 39, 42, 46, 55, and 56) did not inhibit the binding of autoantibodies or most of the IDR specific moabs to TPO (tab. 2). Antibodies to peptide P26, P55, and P56 inhibited to some extent binding of moab 15 to TPO (tab. 2).

It was interesting to test the anti-P14 antibody reaction with truncated fragments of peptide P14 (tab. 3). Truncation of two amino acid from the N- or C-terminal seems to have little influence on anti-peptide antibodies binding to peptide P14, however, further truncation on the N- or C-terminal reduce significantly the antibodies binding. This experiment suggests that antibodies from these rabbits need for the reaction with peptide

Table 2. Inhibition of autoantibodies and mouse monoclonal antibodies (moabs) binding to TPO by anti-peptide P14 antibodies.

| Rabbit and anti-peptide antibody number | Inhibition of antibodies binding to TPO after pre incubation with anti-peptide P14 serum (%)* | | | | | |
|---|---|---|---|---|---|---|
| | Auto-antibodies | Moab 15 (IDR-B) | Moab 18 (IDR-B) | Moab 64 (IDR-B) | Moab 2 (IDR-A) | Moab A4 |
| 1 (P14) | 42 | 98 | 95 | 90 | 8 | 0 |
| 2 (P14) | 39 | 95 | 95 | 92 | 5 | 0 |
| 3 (P14) | 41 | 90 | 92 | 93 | 0 | 0 |
| 4 (P14) | 40 | 92 | 95 | 90 | 6 | 0 |
| 5 (P14) | 0 | 45 | 8 | 10 | 7 | 0 |
| 6 (P14) | 0 | 55 | 12 | 10 | 5 | 0 |
| P26 | 0 | 11 | 0 | 0 | 0 | 0 |
| P39 | 5 | 0 | 0 | 0 | 0 | 0 |
| P42 | 3 | 8 | 0 | 0 | 0 | 0 |
| P46 | 6 | 5 | 0 | 0 | 0 | 0 |
| P55 | 5 | 36 | 0 | 0 | 0 | 0 |
| P56 | 7 | 15 | 0 | 0 | 0 | 0 |

*Standard deviation for all data was between 2 and 5%.

P14 both N- and C-terminal amino acids. Antibodies from rabbit no. 5 and 6 seem to react better with the N-terminal sequence of peptide P14. Anti-peptide antibodies obtained in all six rabbits react only weakly with the middle portion of peptide P14 (peptide 604-612).

The inhibition of conformation-dependent antibodies binding to an antigen by peptide antibodies is frequently ascribed to the effect of steric hindrance (23). Moab 47 reacts with a linear sequence 713-721 (24) of TPO and the steric hindrance effect could be tested experimentally by antibodies to peptides surrounding the epitope for moab 47. Our peptide P1, P22, and P27 are located just around the moab 47 epitope, on the three-dimensional model of TPO (fig. 1) and peptide P4, P6, P20 are slightly detached from the epitope for moab 47 (fig. 2). Antibodies to P43, which covers the epitope for moab 47, inhibited completely the moab 47 binding to TPO (tab. 4). Antibodies to P1, P22, and P27 inhibited the binding of moab 47 to TPO by 25, 10, and 41%, respectively. Part of the amino acid sequence of peptide P18 overlaps the sequence of P1 and the influence of anti-P18 antibodies on moab 47 binding to TPO is similar to antibodies to P1 (results not shown). All other anti-TPO peptide antibodies, covering the whole surface of TPO presented on figure 1 and 2, did not inhibit the binding of moab 47 to TPO (results not shown). Mixtures of antibodies to P1, P22, and P27 inhibited the moab 47 binding to TPO up to 81% (tab. 4). Addition of any other anti-peptide serum to the mixture of anti P1, P22, and P27 did not increase the inhibition of moab 47 binding to TPO. Mixture of the slightly detached peptides, that is P4, P6, and P20, from the moab 47 epitop, inhibit the moab 47 binding to TPO by 24% (tab. 4).

During our work we have noticed increased moab 9 binding to TPO after preincubation of TPO coated plates with some peptide antibodies (tab. 5). Preincubation of TPO coated plates with other peptide antibodies or their mixtures did not influence the binding of moab 9 to such high levels (results not shown). The enhancement of moab 9 binding by some peptide antibodies is in contrast with another IDR-A specific moab 2, where the same peptide antibodies inhibit the binding of moab 2 to TPO by 75% (results not shown).

Table 4. Inhibition of moab 47 binding to TPO by rabbit anti-peptide antibodies and their mixtures.

| **Rabbit antipeptide antibody number** | **Inhibition of moab 47 binding (%)*** |
|---|---|
| P1 | 25 |
| P4 | 0 |
| P6 | 0 |
| P20 | 0 |
| P22 | 10 |
| P27 | 41 |
| P43 | 100 |
| P1 + P22 | 36 |
| P1 + P27 | 70 |
| P1 + P22 + P27 | 81 |
| P4 + P6 + P20 | 24 |
| P1 + P22 + P27 + P4 + P6 + P20 | 79 |

*Standard deviation for all data was between 4 and 6%.

Table 5. Increase of moab 9 binding to TPO after pre-incubation with peptide antibodies.

| **Anti-peptide antibody number** | **Increase of moab 9 binding to TPO (%)*** |
|---|---|
| P12 | 115 |
| P14 | 120 |
| P18 | 110 |
| P43 | 123 |
| P43 + P12 | 270 |
| P43 + P18 | 259 |
| P14 + P12 + P18 | 380 |
| P12 + P14 + P18 + P49 | 512 |

*Standard deviation for all data was between 5 and 10%.

Table 3. Reaction of rabbit antibodies to peptide 14 with peptide fragments of P14.

| **Peptide amino acid sequence** | **Cross reactivity of rabbit antibodies to P14 (599-617) with fragments of peptide P14 (%)*** | | | | | |
|---|---|---|---|---|---|---|
| | **Rabbit 1** | **Rabbit 2** | **Rabbit 3** | **Rabbit 4** | **Rabbit 5** | **Rabbit 6** |
| 601-617 | 90 | 81 | 100 | 89 | 74 | 61 |
| 603-617 | 119 | 54 | 80 | 74 | 68 | 54 |
| 605-617 | 88 | 47 | 42 | 42 | 41 | 1 |
| 607-617 | 24 | 27 | 9 | 39 | 11 | 0.4 |
| 610-617 | 5 | 0.6 | 0.1 | 10 | 0.6 | 1 |
| 599-615 | 118 | 80 | 106 | 65 | 131 | 62 |
| 599-611 | 93 | 75 | 79 | 58 | 116 | 68 |
| 599-609 | 53 | 27 | 57 | 36 | 62 | 800 |
| 599-607 | 17 | 18 | 12 | 3 | 38 | 2.5 |
| 604-612 | 22 | 2 | 10 | 6 | 41 | 2.5 |

*Standard deviation for all data was between 2 and 8%.

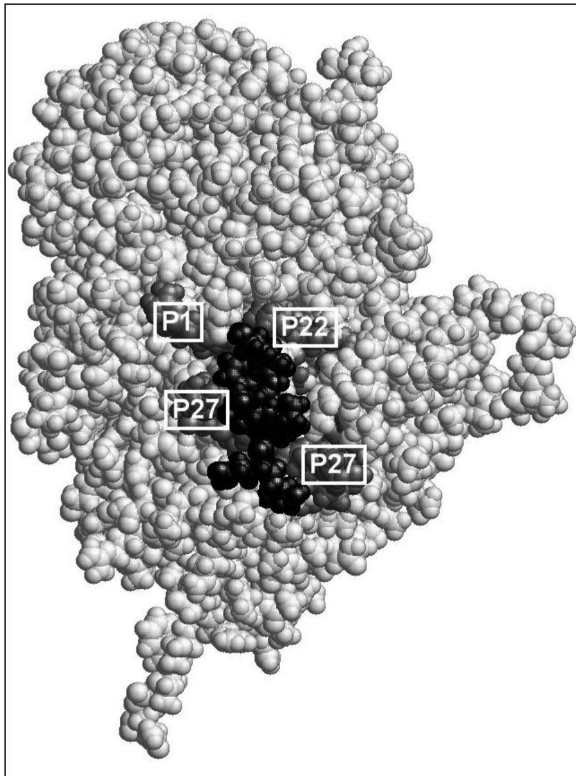


Fig. 1. Localization of moab 47 epitope and neighbouring peptides on the TPO three-dimensional model. Epitope for moab 47 in black, peptides in grey with number of each peptide.

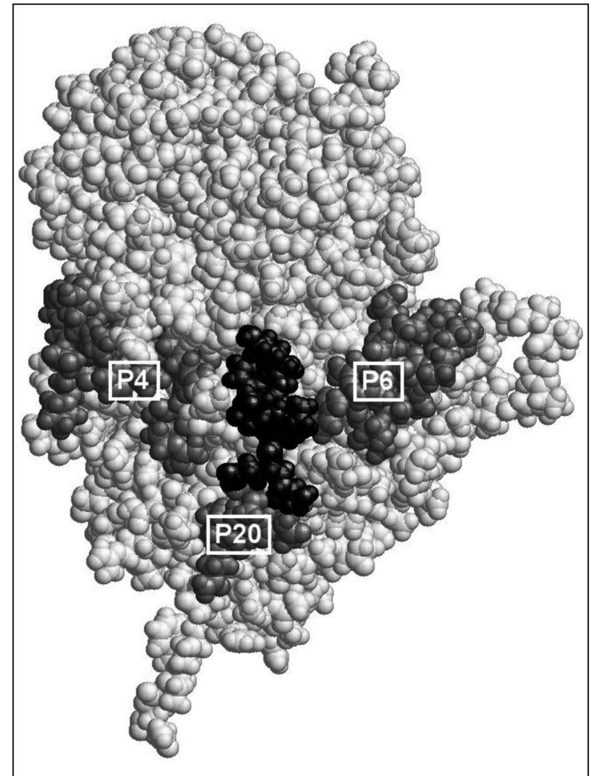


Fig. 2. Localization of moab 47 epitope and peptides slightly detached form the moab 47 epitope on the three-dimensional model of TPO. Epitope for moab 47 in black, peptides in grey with number of each peptide.

Bresson et al. (19) described four amino acid sequences involved in building up the IDR region, namely 353-363, 377-386, 713-720, and 766-775, by replacement of those sequences with the heterogeneous structures. We have obtained antibodies to peptide slightly larger than sequences described by Bresson and co-workers, namely 353-372 (P50), 375-387 (P24), 702-721 (P43), and 760-779 (P54), and all of antibodies to those peptides or their mixtures did not inhibited binding of autoantibodies or IDR-A and -B specific moabs to TPO (results not shown).

## DISCUSSION

The specificity of rabbit peptide antibodies have been questioned by some authors (21, 25, 26). Unexpectedly, majority of our TPO peptide antibodies is highly specific and do not react with human thyroglobulin, human IgG, human albumin, or bovine and egg albumin. Unspecific reaction, if it does occur (peptide P14 reaction with human thyroglobulin), is below 2% of the specific reaction with TPO. It is most probably due to the fact that most of our peptides are rather long and only seven of them are shorter then 10 mer. Reaction of anti-peptide antibodies with human lacto- and myeloperoxidase was expected and it does occur only when the amino acid homology is high. It was not surprising that all of our peptide antibodies react well with denatured TPO on blotting experiments and that most of them react with TPO in ELISA. Seven of anti-peptide sera reacted better with TPO than with the peptide used for immunization. Thus, it suggests that those peptides are better exposed on the TPO surface than coated on polystyrene plates. The possibility of recognition by peptide antibodies (disordered state) of native protein (ordered state) was discussed in the literature (20, 21) and called the order-disorder paradox (27). According to Chersi et al. (21), the reaction of peptide antibodies with native protein is in the range of 10-25%. Indeed, ten of our peptide antibodies do not react with the native protein (P5, P6, P8, P9, P18, P19, P25, P26, P28, P37) and the reaction of other ten is below 25% (P1, P2, P10, P15, P21, P22, P23, P32, P42, P53). However, peptides P1, P5, P8, P25, P28, and P37 are buried or have only a few amino acids exposed on the TPO surface. Peptides P6, P9, P18, and P19 are exposed on the TPO surface and the lack of anti-peptide antibody recognition of native TPO should have another reason. The rest of our peptide antibodies, namely twenty one, react well above 25% and up to 95% with native TPO. Some authors used relative short peptides for immunization (6-8 mer), while our peptides are relatively long (9-20 mer), and this could explain good reaction of our peptide antibodies with native TPO.

It was surprising that anti-peptide antibodies to a relatively short linear amino acid sequence can be used as vaccines and recognize complex native structure in viruses and proteins (28, 29). The reaction of anti-peptide antibodies with native conformational protein structure was ascribed to the cross reactivity (21). Antibodies to our peptide P14 inhibit binding of mouse monoclonal,

human recombinant fab fragments and autoantibodies to TPO (17, 18), directed to conformation-dependent epitopes. This could be interpreted as direct binding to structures involved in the IDR-B region of TPO (17). However, the possible effect of steric hindrance or antibodies induced conformational changes was not examined. Therefore, we have tested these two latter hypotheses by immunization of six rabbits with P14 and a few shorter fragments of P14 and subsequent analysis of obtained sera. Four of these antisera inhibited the IDR-B specific moabs and autoantibodies binding to TPO, as expected, while two of them, to our surprise, do not despite good reaction with TPO. These data suggest that rabbit antibodies directed to the same peptide could recognize different three-dimensional structures on TPO. Analysis of the reaction of anti-P14 antibodies with truncated fragments of P14 indicates that antibodies in rabbit 1 to 4 need for the reaction with P14 both N- and C-terminal amino acids, while antiserum from rabbit no. 5 and 6 seems to react better with the N-terminal sequence. It is possible that recognition of a structure build from C- or N-terminal amino acid of peptide P14 by peptide antibody is required for the inhibition of binding of IDR-B specific antibodies to TPO. This explanation is supported by experiments where antibodies to the N- and C-terminal sequence of peptide P14 (P26, P39, P42, P46, P55, and P56) do not inhibit the binding of autoantibodies to TPO (tab. 2).

An alternative explanation that binding of P14 antibodies induce a conformation changes in the vicinity of P14 is also a possibility, although less likely. Such a conformation changes could effect the structure of a neighbouring regions where the IDR-B is located. The possibility of induced conformational changes by antibodies binding is illustrated by the dramatic increase in moab 9 binding to TPO after preincubation with some peptide antibodies (Tab. 5). This suggests that the epitope of moab 9 is only partially available on the TPO surface and is fully exposed after peptide antibodies binding to TPO. It is interesting that only peptide antibodies that react with the IDRs induce the increase in moab 9 binding to TPO.

The inhibitory effect of peptide antibodies on other antibodies binding to TPO is frequently assign to a steric hindrance effect. The linear epitop for moab 47 is well known (713-721) and we have produced anti-peptide antibodies surrounding the epitope of moab 47 and tested the steric hindrance effect. Only antibodies to peptide P27, whose N-terminal sequence is on one side and C-terminal sequence is on the other side of the epitop for moab 47, significantly (41%) inhibited the moab 47 binding to TPO. Two antibodies to neighbouring sequences, namely P1 and P22, have small effect on moab 47 binding to TPO. The steric hindrance effect is greatly augmented when a mixture of all three peptide antibodies (P1, P22, and P27) was used. Antibodies to peptides slightly detached from moab 47 epitope do not inhibited moab 47 binding to TPO or the inhibition was weak. Increase in inhibition of moab 47 binding to TPO was observed when a mixture of these peptide antibodies (P1, P22, and P27) was used. These data provide an additional argument for the correctness of the previously described TPO model (17). This finding also suggests that steric hindrance of an antibody on binding of another antibody could only be detected if epitopes recognized by both antibodies are very close indeed and even if that happen the effect is not very strong. Strong steric hindrance effect on antibodies binding can be obtained when a mixture of antibodies to neighbouring peptides is used. Any antibody or mixtures of antibodies to peptides separated from the tested epitope have non or small effect on moab 47 binding to TPO. This is perhaps not surprising having in mind the three-dimensional structure of IgG as an oblong molecule with the antigen binding site located at the tip of antibody (30). These experiments support the view that strong inhibition (80%) of autoantibodies binding to TPO obtained by a mixture of peptide antibodies (P12, P14, and P18) described before (18) is an effect of steric hindrance. However, our experiments on the effect of steric hindrance on moab 47 binding to TPO suggest that a strong steric hindrance effect (80% in our case) is a result of close epitope localization on the protein surface. This indicates that the IDR-A should be localized close to the peptide P12, P14 and P18. Strong inhibition of IDR-B specific moabs and human recombinant fabs to TPO by P14 antibodies could not be ascribed to the effect of steric hindrance. It is most probable that at least part of the P14 amino acid sequence is involved in building up the IDR-B region as originally defined by moab 15, 18, and 64 (4). Although the effect of conformational changes induced by P14 antibodies binding to TPO could not be ruled out completely. Our results on the involvement of the P14 amino acid sequence (599-617) in building up the IDR-B are in line with previous finding by others (8, 9, 16) and in apparent disagreement with the results of Bresson et al. (19). Bresson et al. work (19) describe the epitope localization for one human monoclonal antibody (moab T13) in the IDR-A. Our mixture of peptide antibodies (P12, P14, and P18) inhibits IDR-A specific reagent and autoantibodies binding to TPO by the effect of steric hindrance, therefore, the disagreement might be rather apparent than real. Also the absence of competition between our peptide antibodies to sequences described by Bresson et al. (19) and autoantibodies, as negative results do not make a strong argument.

## CONCLUSIONS

Taken together, our data demonstrate that anti-peptide sera generated in our laboratory are a useful tool in research on TPO as an autoantigen. At least part of rabbit sera that were used in the presented study bind to the immunodominant regions on TPO. Thus, some selected peptides seem to be truly the components of the epitopes for human autoantibodies.

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