Loop variants of the serpin thyroxine-binding globulin: implications for hormone release upon limited proteolysis

Helmut GRASBERGER*, Henriette M. B. GOLCHER†, Anja FINGERHUT† and Onno E. JANSSEN†[1]

*Howard Hughes Medical Institute, University of Chicago, 5841 S. Maryland Ave, Chicago, IL 60637, U.S.A., and †Division of Endocrinology, Department of Medicine, University of Essen, Hufelandstrasse 55, 45122 Essen, Germany

Thyroxine-binding globulin (TBG) and corticosteroid-binding globulin are unique among non-inhibitory members of the superfamily of serine-proteinase inhibitors (serpins) in undergoing a dramatic increase in stability [stressed-to-relaxed (S → R) transition] after proteolytic cleavage within their exposed reactive-site-loop (RSL) equivalent. This structural rearrangement involves the insertion of the cleaved loop as a new strand into the β-sheet A and is accompanied by a decrease in hormone binding. To define the mechanism that leads to disruption of hormone binding of TBG after proteolytic cleavage, the effect of partial loop deletions and replacements by the α_1-proteinase inhibitor homologues of TBG were evaluated. Unexpectedly, deletion of the loop's C-terminus, thought to be important for thyroxine binding, improved the binding affinity over that of normal TBG. Proteolytic cleavage of this variant revealed an intact S → R transition and reduced its binding activity to that of cleaved TBG. In contrast, a chimaera with C-terminal loop extension mimicked the decreased binding affinity of cleaved TBG and had a thermal stability intermediate between that of native and cleaved serpins. This variant was still susceptible to loop cleavage and underwent an S → R transition, yet without changing its binding affinity. Our data exclude a direct involvement of loop residues in thyroxine binding of native TBG. Limited insertion of the RSL into β-sheet A appears to trigger hormone release after proteolytic cleavage. In support of this concept, residues within the hinge region of the TBG loop are phylogenetically highly conserved, suggestive of their physiological role as a functional switch *in vivo*.

Key words: chimaera, limited proteolysis, reactive site, targeted hormone delivery, thyroid hormone transport.

INTRODUCTION

Thyroxine-binding globulin (TBG), a 54 kDa glycoprotein of hepatic origin, is the principal carrier of thyroxine (T_4) and triiodothyronine (T_3) in human serum [3]. On the basis of sequence similarity, TBG belongs to the serine-proteinase-inhibitor (serpin) superfamily [4]. Historically, the name serpin is derived from the function of most of their members as inhibitors of serine proteinases. However, some inhibitors of this family target cysteine proteinases, while others have no known inhibitory function. The inhibitory serpins have fundamental importance in the regulation of blood coagulation and fibrinolysis (e.g. antiplasmin, antithrombin III, heparin cofactor II, protein C inhibitor), inflammatory response and extracellular matrix turnover [e.g. α_1-proteinase inhibitor (α_1-PI), α_1-antichymotrypsin, plasminogen-activator inhibitors] and viral pathogenicity (e.g. CrmA, SERP-1) (reviewed in [5]).

The crystallographic structures of several serpins have been resolved (reviewed in [6]). The inhibitory activity of serpins depends on their exposed reactive-site loop (RSL) acting as suicide substrate for their target proteinases. Inhibitory serpins are known to undergo a conformational rearrangement on cleavage, resulting in insertion of the N-terminal part of their RSL in the middle of the underlying β-sheet A and trapping of the bound proteinase as a tight equimolar complex. This structural rearrangement is accompanied by a remarkable increase in thermodynamic stability [stressed-to-relaxed (S → R) transition] [7].

The capacity for loop insertion is thought to be crucial for inhibitory activity and is found in all inhibitory serpins [8,9]. Non-inhibitory serpins, such as ovalbumin [10], angiotensinogen [11], pigment-epithelium-derived factor [12] and maspin [13], show no loop insertion and no S → R transition on cleavage. The hormone-transport proteins TBG and corticosteroid-binding globulin (CBG) also belong to the non-inhibitory serpins; however, they do exhibit an S → R transition and hence loop insertion, as evidenced by their increase in heat stability after cleavage as first described by Pemberton et al. [14]. It has been postulated that this structural rearrangement has evolved for the targeted delivery of their ligands to tissues rich in proteinases, such as sites of inflammation, since cleavage by human leucocyte elastase (HLE) results in disruption of the binding properties of both CBG and TBG. The decrease in the affinity of T_4 binding to TBG by a factor of 3 [15] was less profound than the decrease in cortisol-binding affinity of CBG by a factor of 10 [14], but in agreement with the recently reported increased free-to-bound T_4 ratio of TBG exposed to HLE [16]. While Pemberton et al. [14] were first to describe loss of binding of cleaved CBG, they were

Abbreviations used: CBG, corticosteroid-binding globulin; HLE, human leucocyte elastase; K_a, affinity constant [thyroxine (T_4)-binding]; α_1-PI, α_1-proteinase inhibitor; RSL, reactive-site loop; s, β-strand (e.g. s1C); Sf9, *Spodoptera frugiperda* 9; S → R transition, stressed-to-relaxed transition; serpin(s), serine proteinase inhibitor(s); T_3, tri-iodothyronine; TBG, thyroxine-binding globulin; residues are numbered as described by Schechter and Berger [1], where P1–P1′ is the scissile bond, P2, P3, etc. are residues in the N-terminal direction and P1′, P2′, etc. are residues in the C-terminal direction; for the non-inhibitory TBG, the numbers correspond to the equivalent positions of α_1-PI based on the alignment of the conserved regions preceding and following the RSL respectively; the denotation of serpin secondary-structural elements and their assignment to the TBG sequence are as described in [2].

[1] To whom correspondence should be addressed (e-mail onno.janssen@uni-essen.de).

unable to detect changes in the binding affinity of cleaved TBG. The reason for this discrepancy is unclear, but it could be due to the sensitivity of the method used to detect T_4 release from TBG, since our results have been confirmed as recently shown by Schussler [17]. Indeed, work from Schussler's laboratory showed that the proteolytic cleavage of TBG by leucocytes led to the release of T_4, as determined by equilibrium dialysis [16]. They also reported a rapid disappearance of TBG from serum of subjects undergoing cardiac by-pass surgery, which was not accompanied by a loss of other serum proteins [18,19]. This finding, attributed to TBG consumption due to proteinase cleavage at inflammatory sites, is supported by our finding of loss of T_4-binding capacity due to further degradation of the primary cleaved fragment during exposure to HLE [15]. Finally, Suda et al. [20] probed the ligand-binding site of TBG with a specific fluorophore, 1,8-anilinonaphthalenesulphonic acid, and concluded that T_3 binding also decreases after serine-proteinase cleavage of the TBG molecule.

There is, though, no experimental evidence to establish causality between the ability to undergo the S → R transition and the detrimental effect on the binding properties. Indeed Terry and Blake [21] suggested a direct interaction of residues in the C-terminal part of the RSL with bound T_4, as implied by molecular modelling of the binding site of TBG. These interactions would be disrupted by cleavage within the loop, independently of the concomitant S → R transition.

To better understand the high-affinity T_4 binding and to define the mechanism by which cleavage by HLE leads to a reduction of the binding activity, we have characterized TBG variants by deletions and substitutions of different parts of its RSL by the respective α_1-PI homologues.

MATERIALS AND METHODS

Materials

α_1-PI M-type cDNA [22] was kindly given by Dr R. C. Foreman, School of Biological Sciences, University of Southampton, Southampton, U.K. Vent DNA polymerase and restriction endonucleases were obtained from New England Biolabs. Purified TBG and rabbit anti-TBG serum were generously donated by R. Gärtner, Medizinische Klinik, Klinikum Innenstadt, Department of Endocrinology, Ludwig-Maximilians University, 80336 Munich, Germany. T_4 stock solutions and TBG concentrations were quantified using RIAs [BRAHMS Diagnostica GmbH (Berlin, Germany) and CIS Bio International (Gif-Sur-Yvette, France, now acquired by Schering)].

Construction of hybrid TBG–α_1-PI cDNAs and expression in insect cells

The construction of the TBG transfer vector, the generation of recombinant baculoviruses and the expression in Sf9 (*Spodoptera frugiperda*) insect cells have been described previously [23]. Chimaera TP_{L2} was generated by repeated cycles of two-step PCR overlap extension [24] with TBG and α_1-PI cDNA or the gel-purified intermediate PCR products as templates respectively. The cDNAs were fused sequentially with the internal primers 5′-TCGATGGTCAGCACAGCCTTATGGGCAGCATT-3′ and 5′-AATGCTGCCCATAAGGCTGTGCTGACCATCGA-3′ (antisense and sense primers for N-terminal fusion) and 5′-ATGTCTATCCCCCCCGAAAACACTTTCCTACA-3′ and 5′-TAGGAAAGTGTTTTCGGGGGGGATAGACATGG-3′ (sense and antisense primers for C-terminal fusion). The external primers were 5′-CGGAATTCCTTCCTTCCAAAATGTCA-CC-3′ and 5′-CGGGGTACCCCAATGGCCTTTTTCCCGA-CTA-3′ specific for the N- and C-terminus of the TBG-cDNA, respectively, and 5′-TGAGGAGCGAGAGGCAGTTA-3′ for the C-terminus of the α_1-PI cDNA. These primers provided *Eco*RI and *Kpn*I linkers respectively for subcloning into pBlueBac4 (Invitrogen). The correct sequence of the final product was confirmed by automated sequencing with fluorescent dye terminators (PRISM-System 377, Applied Biosystems).

The shorter loop replacement in chimaera TP_{L1} was constructed via a cassette mutagenesis approach. Briefly, fragments of the human TBG coding sequence were derived from the plasmid pGpT [25]. Fragment A, encoding Met^1 to Ala^{346} of TBG as well as a portion of the cloning vector, was obtained by treatment of pGpT with *Eco*RI and *Pvu*II. Similarly, fragment B, coding for the C-terminal part of TBG starting with Arg^{369}, was obtained by digestion of pGpT with *Hin*dIII and *Bgl*II. Two complementary oligonucleotides (5′-CTGTCCCTTTTTTAGA-GGCCATACCCATGTCTATCCCCCCTATTATCCAAATT-GATA-3′, 5′-GATCTATCAATTTGGATAATAGGGGGGA-TAGACATGGGTATGGCCTCTAAAAAAGGGACAG-3′) coding for residues Phe^{352} to Pro^{361} of α_1-PI flanked by residues Val^{347} to Pro^{348} and Pro^{363} to Asp^{368} of TBG were synthesized and annealed such that the 5′- and 3′-termini of the duplex (fragment C) would be compatible with the 5′-overhang produced by cleavage of pGpT with *Bgl*II and with the blunt-ended recognition site of *Pvu*II respectively. Fragment D was *Eco*RI/*Hin*dIII-digested pGEM-4z (Promega). The ligation was performed in a two-step reaction, with double digestion of the ligation product of fragment A and C with *Eco*RI and *Bgl*II and subsequent ligation of the obtained monomeric ligation product with fragments B and D. The efficiency of this procedure was confirmed by partial sequencing of five ampicillin-resistant JM109 transformants, all of which contained the desired hybrid coding sequence. One clone, designated TP_{L1}, was subcloned into the transfer vector pBlueBac4 and sequenced in its entirety.

Western blotting

Samples were run on continuous Tris/glycine/10 %-(w/v)-polyacrylamide gels under denaturing, non-reducing conditions [26]. For PAGE under non-denaturing conditions, SDS was omitted from all buffers. Blotted nitrocellulose membranes were probed with rabbit anti-TBG antiserum as primary antibody followed by enhanced chemiluminescence (ECL®) immunodetection with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech, Freiburg, Germany) as secondary antibody.

Reaction with HLE

Samples were incubated in assay buffer (0.1 M NaCl/0.05 % Triton X-100/0.1 M Hepes, pH 7.4) with purified HLE (EC 3.4.21.37; Calbiochem) in equimolar concentrations for 20 min at 37 °C. HLE inhibitory activity was determined in the same buffer using methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Calbiochem) as chromogenic substrate as described in [23].

T_4 binding assay

Parameters of T_4 binding to the recombinant proteins were measured by a method previously described in detail [27]. Briefly, samples were diluted with 270 mM barbital buffer, pH 8.6, and incubated with [^{125}I]T_4 (sp. radioactivity 48.8 MBq/μg; DuPont–New England Nuclear) in the presence of increasing amounts of unlabelled T_4. After equilibration, protein bound was separated from free [^{125}I]T_4 with anion-exchange resin beads (M 400;

Mallinckrodt) and the specific ^{125}I-binding determined. The affinity constants (K_a) and binding capacities for T_4 were calculated by Scatchard analysis [28]. The accurate determination by the resin method of the concentration and K_a of TBG both native and cleaved by elastase has recently been verified by equilibrium dialysis [16] and by T_3 displacement [20].

Stability assays

Heat-stability assays was assessed at a temperature range from 40 to 95 °C. Following a 20 min incubation, the samples were rapidly cooled on ice, soluble protein fractionated by centrifugation (15 min at 14000 ***g***) and aliquots loaded on to non-denaturing polyacrylamide gels for analysis. Functional stability was assessed by analysis of residual specific T_4-binding capacity, which was expressed relative to controls kept at 0 °C.

RESULTS

Design of TBG–PI chimaeras

Alignment of the conserved regions preceding and following the hypervariable RSL revealed that TBG and PI differ in the apparent length of this region (Figure 1). Residues near the C-terminus of the TBG loop (namely Glu^{357} and Phe^{360}) have been implicated in the T_4–TBG interaction [21]. Thus two chimaeras differing in the length of their loop sequence were constructed. Chimaera TP_{L1} contained the α_1-PI loop from position P7 to P3′. P7 was fused in-frame to the TBG sequence based on the alignment of the conserved hinge region (P12–P16) of its loop with that of α_1-PI. P3′ was linked to Pro^{363} (the beginning of strand 1C) of TBG. The loop of TP_{L1} was thus four residues shorter than that of normal TBG. In chimaera TP_{L2} a longer PI fragment (P20–P3′) was transferred into the TBG context. P20 was fused in-frame to the aligned TBG sequence and P3′ of the PI fragment was linked to Pro^{356} of TBG. The loop of TP_{L2} was therefore three residues longer than that of normal TBG. Both chimaeras and TBG were expressed in Sf9 cells using recombinant baculovirus. All recombinant proteins were secreted at similar expression levels of up to 5 μg/ml as determined radioimmunometrically and by Scatchard analysis of T_4 binding (results not shown).

Reaction with HLE

Both chimaeras were specifically cleaved by HLE (Figure 2), but cleavage of TP_{L2} was much slower than that of TBG and TP_{L1} (results not shown). The apparent molecular masses of the reaction products were compatible with cleavage within their RSL. Like normal TBG, both chimaeras reacted as pure substrates and not as inhibitors, since no complex formation with HLE was observed (Figure 2) and no measurable HLE inhibition was found in an inhibitor assay (results not shown).

```
          P16  P12          P1/P1'
TBG  339  EKGTEAAAVPEVELSDQ---PENTFLHPIIQID  368
PI   342  EKGTEAAGAMFLEAIPMSIP----------PEVKFN  367
TPL1 339  EKGTEAAAVPFLEAIPMSIP--------PIIQID  364
TPL2 339  EKGTEAAGAMFLEAIPMSIPPENTFLHPIIQID  371
          ◄──────── RSL ────────►◄ s1C ─
```

Figure 1 Alignment of the RSL of TBG, α_1-PI and the chimaeras

Parts of the amino acid sequences of TBG, α_1-PI and the two chimaeras are shown. The numbers to the left and right indicate the N- and C-terminal positions of the amino acid sequences. The reactive site is indicated by P1/P1′. P12–P16 represent the loop's hinge region. The secondary-structure elements of the α_1-PI model are indicated at the bottom. Shaded areas indicate residues taken from the α_1-PI sequence.

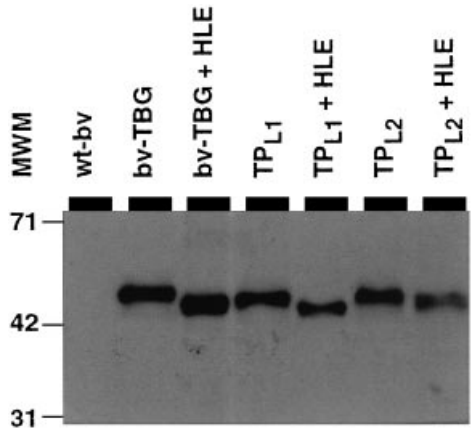

Figure 2 Reaction of recombinant serpins with HLE

Recombinant TBG and the chimaeras were incubated either alone (−) or with (+) an equimolar amount of HLE for 30 min at 37 °C. The reaction products were separated by non-reducing SDS/PAGE. After incubation with HLE, all proteins showed a decrease in apparent molecular mass consistent with specific cleavage within their RSL. No SDS-stable complexes with HLE, which would have been indicative of true inhibition, were detected.

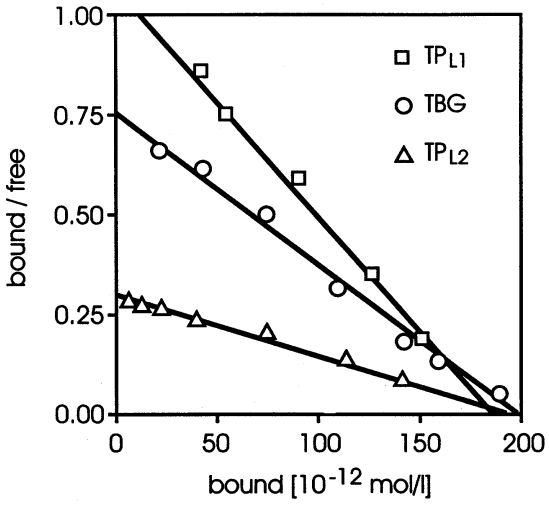

Figure 3 Effect of loop length on T_4 binding

Changes of the TBG loop markedly influenced the T_4-binding properties: the affinity constant (K_a) for T_4 binding of TP_{L2} (△) was reduced to one-third of that of TBG (○) [$(0.38 \pm 0.16) \times 10^{10}$ M^{-1} versus $(1.17 \pm 0.19) \times 10^{10}$ M^{-1}, means ± S.D.]. In contrast, TP_{L1} (□) had a higher binding affinity [K_a $(1.69 \pm 0.21) \times 10^{10}$ M^{-1}] than that of TBG. Plots shown are representative of four independent experiments.

Analysis of T_4 binding

The effect of loop exchange on T_4 binding was assessed with a resin uptake test and Scatchard analysis. Unexpectedly, substitution of the loop by the α_1-PI homologue had a pronounced effect on the T_4 binding properties: The binding affinity of TP_{L2} at a K_a of $(0.38 \pm 0.16) \times 10^{10}$ M^{-1} was only one-third of that of native TBG [K_a $(1.17 \pm 0.19) \times 10^{10}$ M^{-1}] (Figure 3), similar to

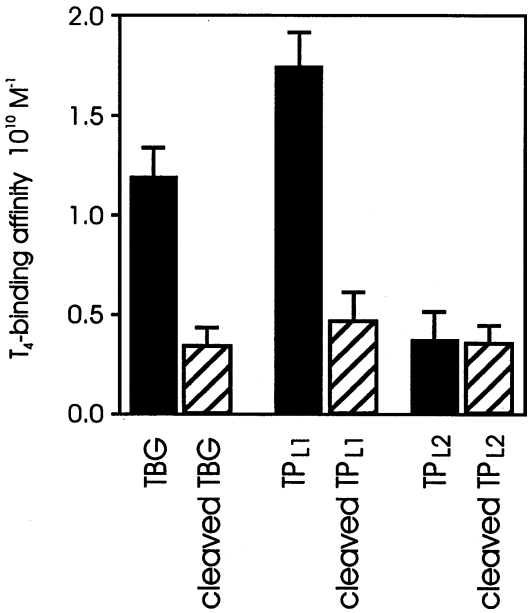

Figure 4 Effect of HLE cleavage on T_4 binding

Affinity constants (K_a) for T_4 binding were determined before (black bars) and after (hatched bars) specific cleavage with HLE (means ± S.D. for four independent experiments).

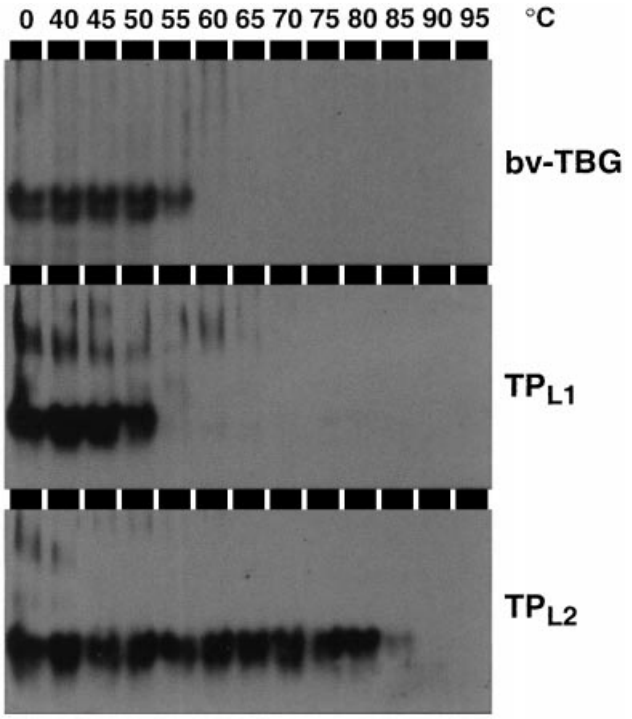

Figure 5 Thermal denaturation of recombinant serpins

TBG and the chimaeras were incubated for 20 min at the indicated temperatures, separated by non-denaturing PAGE and probed with anti-TBG antiserum. TP_{L2} showed neither signs of polymerization nor loss of detectable antigen at temperatures as high as 85 °C. TBG and TP_{L1} were stable only up to temperatures of 60 °C. bv-TBG, TBG synthesized in the baculovirus system described in the Materials and methods section.

that of cleaved TBG (see below). In contrast, TP_{L1} had a higher T_4-binding affinity than normal TBG [K_a $(1.69 \pm 0.21) \times 10^{10}$ M^{-1}], thus representing the first protein with a higher T_4-binding affinity than TBG.

Cleavage of their RSL reduced the T_4-binding affinity of TBG and TP_{L1} [to K_a values of $(0.33 \pm 0.11) \times 10^{10}$ M^{-1} and $(0.44 \pm 0.17) \times 10^{10}$ M^{-1} respectively], whereas cleavage of TP_{L2} did not further decrease its T_4-binding affinity (Figure 4). Thus native and cleaved TP_{L2} resembled the functional properties of cleaved TBG.

Stability assays

To assess the conformational stability of the recombinant proteins, their soluble fraction after heat denaturation was separated on native PAGE. TBG and TP_{L1} were denatured and lost their ability to migrate as monomers after incubation at 55 and 50 °C respectively (Figure 5). In contrast, TP_{L2} exhibited neither multimerization nor loss of soluble antigen at temperatures as high as 80 °C. With this increased stability, native TP_{L2} almost mimicked the stability profile of the relaxed (R) conformation of serpins.

To test if the variants underwent an S → R transition upon cleavage, the functional stability of their native and cleaved forms was assessed by determination of their residual activity after thermal denaturation (Figure 6). Native TP_{L1} was inactivated at a slightly lower temperature than TBG (55 versus 60 °C). In accordance with the results of the native PAGE, T_4 binding of TP_{L2} was remarkably heat-resistant, with no significant loss after incubation at temperatures as high as 80 °C for 20 min. After HLE cleavage, all recombinant proteins had an increased functional stability, i.e. all three proteins were converted into a more relaxed state by cleavage within their RSL.

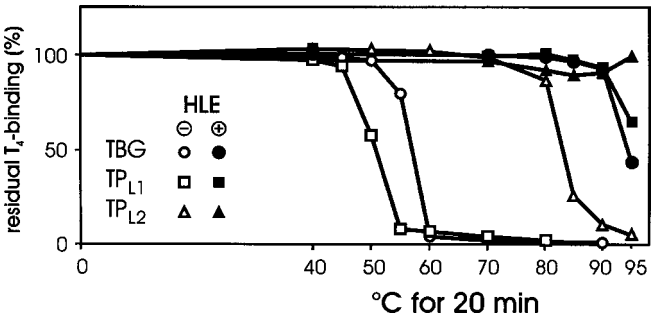

Figure 6 Thermal inactivation profile of intact and cleaved recombinant serpins

Aliquots of the intact and HLE-cleaved recombinant proteins were incubated between 40 °C and 95 °C for 20 min. Soluble proteins were then fractionated by centrifugation and analysed for their residual T_4-binding capacity, which is expressed relative to control samples kept at 0 °C (mean values for duplicate experiments). Open symbols mark uncleaved samples while the corresponding filled symbols mark HLE-cleaved proteins. Intact TP_{L1} (□) was slightly less stable than TBG (○), but after cleavage, it (■) was as stable as HLE-cleaved TBG (●). TP_{L2} (△) showed a markedly increased thermal stability with no loss of T_4-binding capacity at temperatures of up to 85 °C. After cleavage its (▲) stability further increased, indicating an intact S → R transition.

DISCUSSION

The hormone-binding serpins TBG and CBG have both been implicated in the targeted delivery of their ligands to tissues rich in proteinases, e.g. sites of inflammation [14–16,20]. The dis-

ruption of binding is caused by the specific proteolytic cleavage within a surface loop, which aligns to the RSL of inhibitory serpins. During this process their exposed loop is believed to undergo a drastic conformational change, with insertion of the part N-terminal to the cleavage site into the major β-pleated sheet A. Indirect evidence for this is inferred from the characteristic increase in stability following cleavage (S → R transition), which is observed in all inhibitory serpins. Crystallography of their cleaved forms shows insertion of the N-terminal loop as a new strand (strand 4A) into β-sheet A.

Alignment of serpin sequences (Figure 1) suggests that TBG has a relatively long loop conformation, four residues (Glu^{357}–Phe^{360}) longer than the respective α_1-PI sequence. However, the RSL of serpins is quite variable in length, with antichymotrypsin sharing a similar conformation to TBG in this region. On the basis of an alternative alignment, others have modelled these residues as a β-bulge within the following strand 1C [21]. This was postulated to provide increased access to the underlying β-barrel structure comprising the T_4-binding site [23]. In addition, an interaction of these residues with the iodine atoms of T_4 via Phe^{360} and with the α-amino group of T_4 via Glu^{357} has been postulated.

To examine the importance of the loop in T_4 binding of TBG, we designed chimaeric TBG–α_1-PI molecules by replacing the loop with its α_1-PI counterpart, with and without deleting the additional residues of TBG. Contrary to the prediction, the deletion of Glu^{357}–Phe^{360} resulted in a variant (TP_{L1}) with an even higher T_4-binding affinity than normal TBG (Figure 3). These data essentially excluded the functional importance of the deleted residues for T_4 binding.

While the transfer of the PI RSL failed to confer HLE-inhibitory activity to TP_{L1}, the molecule still underwent the typical S → R transition after cleavage by HLE (Figure 6). The lack of HLE inhibition by TP_{L1} was thus not due to the inability of the α_1-PI loop to insert into β-sheet A of TBG *per se*. In addition, the concomitant reduction in T_4-binding affinity (Figure 4) clearly showed that the disruption of T_4 binding after cleavage of TBG does not depend on the presence of a TBG-specific loop sequence.

To elucidate whether subtle global structural rearrangements or a more specific interaction at the loop's hinge account for the decreased binding affinity of cleaved TBG, we constructed a second chimaera including the additional, TBG-specific residues (Figure 1). Extension of the loop by three residues resulted in a 3-fold reduction of T_4-binding affinity when compared with native TBG and thus in a binding affinity similar to cleaved TBG (Figures 3 and 4). TP_{L2} exhibited a remarkable thermostability, with neither loss of binding nor polymerization at temperatures of up to 80 °C (Figures 5 and 6).

The dramatic increase in stability of TP_{L2} can be explained by detachment of strand 1C from β-sheet C, a mechanism by which inactivation by polymerization of other serpins take place [29]. Extending the loop could retard this process in that it allows the stronger annealing of strand 1C to β-sheet C, as has been recently proposed for a thermostable α_1-PI variant with a C-terminally extended loop [30]. Such a model, though, would be difficult to reconcile with the reduced binding affinity of TP_{L2}, which rather suggests a conformational change in the β-barrel [23]. Alternatively, TP_{L2}'s thermostability could also be explained by a latent conformation similar to native plasminogen-activator inhibitor-1 [31], in which complete loop insertion occurs by simultaneous detachment of strand 1C from β-sheet C [32]. However, inconsistent with a latent conformation, the TP_{L2} loop was still susceptible to HLE cleavage. Taken together, our data are compatible with partial pre-insertion of the TP_{L2} loop, sufficient to cause diminished T_4 binding. Superimposing the native and cleaved serpin structures, e.g. of α_1-PI, reveals the lateral displacement of strand 3A and expansion of β-sheet A upon loop insertion [33]. Similarly, in TP_{L2}, modelling of partial loop insertion reveals a lateral dislocation of the C-terminal part of the neighbouring strand 3A, residues of which are facing into the interior of the β-barrel motif. Thus, in keeping with the results of recent studies mapping the T_4–ligand binding site by site-directed mutagenesis [34] and characterization of the binding-deficient variant TBG-Aborigine [35], where an Ala^{191} → Thr replacement at the end of strand 3A results in diminished T_4 binding, we hypothesize that disruption of T_4 binding in TP_{L2} is due to weakening of interactions of T_4 with residues in the close vicinity of the loop's hinge region. Consistent with this explanation, complete loop insertion after cleavage did not alter T_4 binding of TP_{L2} (Figure 4). In addition, cleavage of the RSL should further increase heat stability by completion of an energetically favoured antiparallel β-sheet A, and this was, indeed, the case for TP_{L2} (Figure 6). This hypothesis also explains the puzzling finding of an increased T_4-binding affinity in TP_{L1}, assuming a pre-insertion of the loop in normal TBG. The shorter loop of TP_{L1} would result in reduced pre-insertion of its loop, which would then lead to the observed increase in T_4 binding and reduction in stability of this chimaera.

Further credence to the concept that hormone release of TBG after cleavage critically depends on the insertion of only the N-terminal part of its loop stems from an alignment of multiple serpin RSLs, which reveals conservation only of the hinge region (P12–P16) of the TBG loop between species as well as to the consensus sequence of inhibitory serpins. This conservation suggests a functional importance of the structural flexibility of this region in TBG. Since receptor-mediated uptake of cleaved TBG has not been substantiated [36], the ligand-releasing function is the more likely candidate for facilitated hormone transport [15,16,20].

In conclusion, our results offer a model of how the metastability of inhibitory serpins might have been adapted in the non-inhibitory serpin TBG to serve as a targeted hormone-releasing mechanism. Accordingly, partial insertion of the reactive site loop of TBG in β-sheet A is sufficient to trigger hormone release, presumably by a local structural rearrangement in the vicinity of the loop's hinge region in keeping with the previously identified ligand-binding site [23,34]. The remarkable conservation of residues within the hinge region of TBG not only lends support to our model, but also suggests a physiological role of this evolutionary conserved functional switch *in vivo*.

This work was supported by grants from the Deutsche Forschungsgemeinschaft to O.E.J. (DFG Ja 671/1-3 and SFB 469/B8).

REFERENCES

1 Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. papain. Biochem. Biophys. Res. Commun. **27**, 157–162
2 Huber, R. and Carrell, R. W. (1989) Implications of the three-dimensional structure of α_1-antitrypsin for structure and function of serpins. Biochemistry **28**, 8951–8966
3 Refetoff, S. (1989) Inherited thyroxine-binding globulin abnormalities in man. Endocr. Rev. **10**, 275–293
4 Flink, I. L., Bailey, T. J., Gustafson, T. A., Markham, B. E. and Morkin, E. (1986) Complete amino acid sequence of human thyroxine-binding globulin deduced from cloned DNA: close homology to the serine antiproteases. Proc. Natl. Acad. Sci. U.S.A. **83**, 7708–7712
5 Potempa, J., Korzus, E. and Travis, J. (1994) The serpin superfamily of proteinase inhibitors: structure, function, and regulation. J. Biol. Chem. **269**, 15957–15960
6 Whisstock, J., Skinner, R. and Lesk, A. M. (1998) An atlas of serpin conformations. Trends Biochem. Sci. **23**, 63–67

7 Bruch, M., Weiss, V. and Engel, J. (1988) Plasma serine proteinase inhibitors (serpins) exhibit major conformational changes and a large increase in conformational stability upon cleavage at their reactive sites. J. Biol. Chem. **263**, 16626–16630

8 Carrell, R. W. and Owen, M. C. (1985) Plakalbumin, α_1-antitrypsin, antithrombin and the mechanism of inflammatory thrombosis. Nature (London) **317**, 730–732

9 Evans, D. L., McGrogan, M., Scott, R. W. and Carrell, R. W. (1991) Protease specificity and heparin binding and activation of recombinant protease nexin I. J. Biol. Chem. **266**, 22307–22312

10 Gettins, P. (1989) Absence of large-scale conformational change upon limited proteolysis of ovalbumin, the prototypic serpin. J. Biol. Chem. **264**, 3781–3785

11 Stein, P. E., Tewkesbury, D. A. and Carrell, R. W. (1989) Ovalbumin and angiotensinogen lack serpin S–R conformational change. Biochem. J. **262**, 103–107

12 Becerra, S. P., Sagasti, A., Spinella, P. and Notario, V. (1995) Pigment epithelium-derived factor behaves like a noninhibitory serpin. Neurotrophic activity does not require the serpin reactive loop. J. Biol. Chem. **270**, 25992–25999

13 Pemberton, P. A., Wong, D. T., Gibson, H. L., Kiefer, M. C., Fitzpatrick, P. A., Sager, R. and Barr, P. J. (1995) The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. Evidence that maspin is not a protease inhibitory serpin. J. Biol. Chem. **270**, 15832–15837

14 Pemberton, P. A., Stein, P. E., Pepys, M. B., Potter, J. M. and Carrell, R. W. (1988) Hormone binding globulins undergo serpin conformational change in inflammation. Nature (London) **336**, 257–258

15 Janssen, O. E., Golcher, H. M. B., Grasberger, H., Saller, B., Mann, K. and Refetoff, S. (2002) Characterization of thyroxine-binding globulin cleaved by human leukocyte elastase. J. Clin. Endocrinol. Metab. **87**, 1217–1222

16 Jirasakuldech, B., Schussler, G. C., Yap, M. G., Drew, H., Josephson, A. and Michl, J. (2000) A characteristic serpin cleavage product of thyroxine-binding globulin appears in sepsis sera. J. Clin. Endocrinol. Metab. **85**, 3996–3999

17 Schussler, G. C. (2000) The thyroxine-binding proteins. Thyroid **10**, 141–149

18 Afandi, B., Schussler, G. C., Arafeh, A. H., Boutros, A., Yap, M. G. and Finkelstein, A. (2000) Selective consumption of thyroxine-binding globulin during cardiac bypass surgery. Metabolism Clin. Exp. **49**, 270–274

19 Jirasakuldech, B., Schussler, G. C., Yap, M. G., Zirkind, R., Afandi, B. and Michl, J. (2001) Cleavage of thyroxine-binding globulin during cardiopulmonary bypass. Metabolism Clin. Exp. **50**, 1113–1116

20 Suda, S. A., Gettins, P. G. and Patston, P. A. (2000) Linkage between the hormone binding site and the reactive center loop of thyroxine binding globulin. Arch. Biochem. Biophys. **384**, 31–36

21 Terry, C. J. and Blake, C. C. (1992) Comparison of the modelled thyroxine binding site in TBG with the experimentally determined site in transthyretin. Protein Eng. **5**, 505–510

22 Ciliberto, G., Dente, L. and Cortese, R. (1985) Cell-specific expression of a transfected human α_1-antitrypsin gene. Cell **41**, 531–540

23 Grasberger, H., Buettner, C. and Janssen, O. E. (1999) Modularity of serpins. A bifunctional chimera possessing alpha1-proteinase inhibitor and thyroxine-binding globulin properties. J. Biol. Chem. **274**, 15046–15051

24 Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. and Pease, L. R. (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77**, 61–68

25 Janssen, O. E. and Refetoff, S. (1992) *In vitro* expression of thyroxine-binding globulin (TBG) variants. Impaired secretion of TBGPRO-227 but not TBGPRO-113. J. Biol. Chem. **267**, 13998–14004

26 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**, 680–685

27 Murata, Y., Refetoff, S., Sarne, D. H., Dick, M. and Watson, F. (1985) Variant thyroxine-binding globulin in serum of Australian aborigines: its physical, chemical and biological properties. J. Endocrinol. Invest. **8**, 225–232

28 Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**, 660–672

29 Chang, W. S., Whisstock, J., Hopkins, P. C., Lesk, A. M., Carrell, R. W. and Wardell, M. R. (1997) Importance of the release of strand 1C to the polymerization mechanism of inhibitory serpins. Protein. Sci. **6**, 89–98

30 Bottomley, S. P. and Chang, W. S. (1997) The effects of reactive centre loop length upon serpin polymerisation. Biochem. Biophys. Res. Commun. **241**, 264–269

31 Kvassman, J. O., Lawrence, D. A. and Shore, J. D. (1995) The acid stabilization of plasminogen activator inhibitor-1 depends on protonation of a single group that affects loop insertion into β-sheet A. J. Biol. Chem. **270**, 27942–27947

32 Mottonen, J., Strand, A., Symersky, J., Sweet, R. M., Danley, D. E., Geoghegan, K. F., Gerard, R. D. and Goldsmith, E. J. (1992) Structural basis of latency in plasminogen activator inhibitor-1. Nature (London) **355**, 270–273

33 Stein, P. and Chothia, C. (1991) Serpin tertiary structure transformation. J. Mol. Biol. **221**, 615–621

34 Buettner, C., Grasberger, H., Hermansdorfer, K., Chen, B., Treske, B. and Janssen, O. E. (1999) Characterization of the thyroxine-binding site of thyroxine-binding globulin by site-directed mutagenesis. Mol. Endocrinol. **13**, 1864–1872

35 Takeda, K., Mori, Y., Sobieszczyk, S., Seo, H., Dick, M., Watson, F., Flink, I. L., Seino, S., Bell, G. I. and Refetoff, S. (1989) Sequence of the variant thyroxine-binding globulin of Australian aborigines. Only one of two amino acid replacements is responsible for its altered properties. J. Clin. Invest. **83**, 1344–1348

36 Robbins, J. (1996) Thyroid hormone transport proteins and the physiology of hormone binding. In The Thyroid, vol. 7 (Braverman, L. E. and Utiger, R. D., eds.), pp. 96–110, Lippincott–Raven Publishers, Philadelphia

Received 3 January 2002/4 March 2002; accepted 3 April 2002
Published as BJ Immediate Publication 3 April 2002, DOI 10.1042/BJ20020014