Modularity of Serpins

A BIFUNCTIONAL CHIMERA POSSESSING $\alpha_1$-PROTEINASE INHIBITOR AND THYROXINE-BINDING GLOBULIN PROPERTIES

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An exciting application of protein engineering is the creation of proteins with novel functions by the retrofitting of native proteins. Such attempts might be facilitated by the idea of a mosaic architecture of proteins out of structural units. Even though numerous theoretical concepts deal with the delineation of structural “modules,” their potential in the design of proteins has not yet been sufficiently exploited. To address this question we used a gain of function approach by designing modular chimeric molecules out of two structurally homologous but functionally diverse members of the superfamily of serine-proteinase inhibitors, $\alpha_1$-proteinase inhibitor and thyroxine-binding globulin. Substitution of two of four $\alpha_1$-proteinase inhibitor modules (Lys$^{225}$ to Leu$^{308}$ and Pro$^{362}$ to Lys$^{494}$, respectively), identified by $\alpha$-backbone distance analysis, with their thyroxine-binding globulin homologues resulted in a bifunctional chimera with inhibition of human leukocyte elastase and high affinity thyroxine binding. To our knowledge, this is the first report on a bifunctional chimera engineered from modules of homologous globular proteins. Our results demonstrate how a modular concept can facilitate the design of new functional proteins by swapping structural units chosen from members of a protein superfamily.

In all but the smallest proteins, crystallography has revealed that polypeptide chains form several more or less compact units. When loosely connected to the remaining molecule, such units are usually referred to as domains, which implicates the possibility of an autonomous existence (1). In many other cases, the mosaic nature of proteins is less obvious, and numerous concepts have been developed to facilitate the delineation of “modules” thought to rule the folding, function, and biological evolution of proteins (2–8). The increasing frequency with which functionally unrelated proteins are found to contain recurrent structural motifs suggests that the number of natural folds is limited (9, 10) and that complex proteins have evolved by modular assembly (11). Such evolutionarily refined structural units are attractive candidates as building blocks for the design of novel proteins. This concept may be exploited for the in vitro recombination of homologous, i.e. structurally related, proteins.

Based on sequence similarities, an ever increasing number of homologous but functionally diverse proteins are recognized as members of the superfamily of serine-protease inhibitors (serpins).1 They presumably evolved from a common ancestor at least 500 million years ago (12). Most of more than 100 known members of the serpin superfamily are true inhibitors of serine proteinases, best exemplified by the archetypical serpin $\alpha_1$-proteinase inhibitor ($\alpha_1$PI). Serpins are fundamentally important in the regulation of major proteolytic cascades, such as blood coagulation, fibrinolysis, inflammatory response, and extracellular matrix turnover (reviewed in Ref. 13). However, some serpins have lost the inhibitory function and serve as transport proteins for small ligands, such as thyroxine-binding globulin (TBG) (14) and corticosteroid-binding globulin (15). TBG has an exceptionally high binding constant ($K_B = 10^{10}$ M$^{-1}$) for thyroxine ($T_4$) and a binding energy close to a covalent bond (16).

The crystallographic structures of several serpins have been determined (reviewed in Refs. 17 and 18). Their highly compact single-domain structure has a scaffold of three crossed $\beta$-sheets (A–C). Inhibitory serpins are characterized by a reactive site loop (RSL) located between $\beta$-sheets A and C. Proteinase inhibition involves the incorporation of the cleaved RSL into the A-sheet. This structural rearrangement is accompanied by an increase in stability (stressed-to-relaxed transition (19)) and the generation of SDS-stable serpin-proteinase complexes (20). Although the individual serpins have become remarkably diversified by evolution, they share a common molecular pathology (21). Inhibitory dysfunction is caused by disturbances of the hinges of the RSL (P14–P12 of the RSL and strand 1C) (22) or by prevention of insertion (23).

Although $\alpha_1$PI has no known ligand, its sheet C and part of sheet B form a twisted $\beta$-barrel-like structure, characteristic of ligand-binding proteins. By affinity labeling the homologous regions have been shown to comprise the hormone-binding sites of TBG (24) and corticosteroid-binding globulin (25), both of which share 40% sequence identity with $\alpha_1$PI.

So far, it has not been tested whether the inhibitory function and the ligand-binding function are mutually exclusive within the serpin scaffold. We now present a novel concept of a modular architecture of the serpin structure and construction of an $\alpha_1$PI-TBG chimera with both inhibitory activity and high affinity $T_4$ binding.

1) The abbreviations used are: serpin, serine-proteinase inhibitor; $\alpha_1$PI, $\alpha_1$-proteinase inhibitor; HLE, human leukocyte elastase; PAGE, polyacrylamide gel electrophoresis; TBG, thyroxine-binding globulin; RSL, reactive site loop; SI, stoichiometry of inhibition; $T_4$, thyroxine; s, strand (i.e. s1C).

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**TABLE I**

Oligonucleotide primers for splicing by overlap extension polymerase chain reaction

For the internal primers, the first letter of the primer names denotes their 5′-origin from the TBG or α1PI sequence. H1, H2, and R denote the locations of the corresponding splicing sites (bold letters) at homology regions H1 (α1PI numbering: Val206–Met221), H2 (Pro289–Thr294), and the RSL (Pro210–Thr215), respectively. N or C denote N- or C-terminal external primers, specific for the TBG or α1PI coding sequences or their reverse complements, respectively (bold letters).

| Name    | Sequence             | Sense/antisense |
|---------|----------------------|-----------------|
| Internal primers |                        |                 |
| P-H1    | 5′-ACC GTC CGG TTC TCA AAAG | S               |
| T-H1    | 5′-CAT CGG CCA TCA | A               |
| P-H2    | 5′-GCT AGC GAA AAA GGA | A               |
| T-H2    | 5′-TTG ATT CCT | A               |
| P-RS    | 5′-ATG TCT TCT CGG GGA | S               |
| T-RS    | 5′-TAG GAA GTT TCT GGG GGG GAT | A               |
| External primers |                      |                 |
| T-N     | 5′-GTCCTGTCCA AAA ATG TCA CC | S               |
| T-C     | 5′-CGGGCTACCCCAAATGGCCTTTTCCCCGA CTA | A               |
| P-N     | 5′-GATCTGACGAGCAATGACGGA CTA | S               |
| P-C     | 5′-TGAGAGCCAGGAGGACAG CTA | A               |

**EXPERIMENTAL PROCEDURES**

Materials—α1PI M-type cDNA (26) was a kind gift from R. Foreman (Southampton, United Kingdom). A vector containing the full-length cDNA of TBG had been constructed previously (27). Vent DNA polymerase and restriction endonucleases were obtained from New England Biolabs. Spodoptera frugiperda Sf9 cells (ATCC catalog no. CRL 1711) and wild type baculovirus DNA were from Invitrogen. Liposomes for transfection and SF900 II insect cell medium were purchased from Life Technologies, Inc. Purified TBG and rabbit anti-TBG serum were generously donated by R. Gartner (Munich, Germany). Rabbit anti-α1PI serum, human leukocyte elastase (HLE, EC 3.4.21.37), and transthyretin were from Calbiochem. T9 stock solutions and TBG concentrations were quantified using commercially available radioimmunoassays (Brahms Diagnostica, Berlin, Germany and CIS Bio Int. Gif-Sur-Yvette, France). Inhibition assays and active site titrations were measured on a Beckman DU 640 spectrophotometer.

Construction of Hybrid TBG-α1PI Transfer Vectors—Human TBG cDNA was subcloned via the Kpn1 and HindIII sites and human α1PI cDNA via the PstI site into the transfer vector pBlueBac4 (Invitrogen). The splicing sites of the chimeras mapped to highly conserved regions (homology region H1 and H2) and to a putative permissive surface loop (splicing site RS, C-terminal to the RSL). The chimeric constructs were then generated by repeated cycles of two-step polymerase chain reaction overlap extension (28) with the linearized TBG and α1PI plasmids or the gel-purified intermediate polymerase chain reaction products (P1T2+P3−4, P1T2P3−4) as templates, respectively. The cDNAs were ligated to a derestricted pBlueBac4 vector. The correct sequence of the final products was confirmed by automated sequencing with fluorescent dye terminators (PRISM System 377, Applied Biosystems).

Generation of Recombinant Baculovirus and Expression in Insect Cells—Sf9 cells (5 × 10^6 log phase) maintained exclusively in serum-free medium were cotransfected with 1 μg of linearized wild type virus and 4 μg of each of the transfer plasmids by lipofection (29). β-Galactosidase-positive recombinant clones were selected by plaque assay and screened for wild type virus contamination by polymerase chain reaction (30). For protein expression, log-phase Sf9 cells from a spinner culture were seeded in tissue culture flasks and infected with recombinant virus at a multiplicity of infection of five. The medium was changed 12 h later and supplemented with 10 μM 1-t-trans-epoxy-epoxycyclinuclycine-(4′)-guanidinobutane and 10 μM pepstatin A (both from Roche Molecular Biochemicals). Forty-eight hours post infection, the culture supernatants were collected by centrifugation at 1500 × g for 15 min, concentrated, and washed (0.1% NaCl, 0.1% Heps, pH 7.4) by ultrafiltration (Centrisep 30, Millipore Corp.). Protein concentrations were determined by Scatchard analysis of T9 binding and by densitometry of Coomassie Blue-stained gels using purified serum TBG as the standard.

Western Blotting—Samples were run on 10% continuous tris/glycine gels under denaturing, nonreducing conditions. For PAGE under native conditions, SDS was omitted from all buffers. Blotted nitrocellulose membranes were probed with rabbit anti-TBG or rabbit anti-α1PI antisera as primary antibody, respectively, followed by enhanced chemiluminescence immunodetection with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) as secondary antibody.

Inhibitor Assay—HLE was incubated at 37 °C for 15 min with increasing amounts of recombinant proteins in assay buffer (see above). The residual proteolytic activity was calculated from the decrease in absorbance (410 nm) after the addition of 0.5 μM 125I-N-methoxysuccinyl-(A-A-P-V-p-nitroanilide (Calbiochem) as chromogenic substrate. Rates of substrate hydrolysis were constant over the 3-min period used to determine residual activities. The intercept on the abscissa of the plot of the fraction of enzyme remaining (E/Eo) versus the ratio of the initial inhibitor to initial enzyme concentration (I0/Eo) yielded the apparent stoichiometry of the reaction. Control reactions with supernatants of cells infected with baculovirus expressing TBG excluded endogeneous HLE. Inhibitory activity, degradation of HLE, and substrate loss to endogenous proteases.

T9 Binding Assay—Parameters of T9 binding to the recombinant proteins were measured by a method previously described in detail (31). Briefly, samples were diluted with 270 mM barbital buffer (pH 8.6) or phosphate-buffered saline (pH 8.0) and incubated with [125I]T9 (specific activity, 48.8 MBq/μg, NEN Life Science Products) in the presence of increasing amounts of unlabeled T9. After equilibration, protein bound was separated from free [125I]T9 with anion exchange resin beads (M-400, Mallinkrodt), and the specific 125I binding was determined. The affinity constants (Kd) and binding capacities for T9 were calculated by Scatchard analysis (32).

Heat Denaturation—The functional stability of recombinant proteins was quantified by thermal denaturation in a water bath at 60 ± 1 °C for various periods of time or at various temperatures for 20 min, respectively. The samples were then cooled on ice and centrifuged for 15 min at 13,000 × g to remove precipitated protein. Residual specific T9 binding capacity or HLE inhibitory activity was expressed relative to controls kept at 4 °C. The half-lives (t1/2) of heat denaturation were calculated by least square analysis of semi-logarithmic plots of the remaining specific T9 binding versus time of incubation.

**RESULTS**

**Design and Construction of Chimeras**—Based on the structure of α1PI and guided by a distance analysis of its carbon backbone using a diagonal plot (8, 33), four compact structural units of the serpin fold were identified (Figs. 1 and 2A). Modules 1 and 3 complement each other to form an α-β sandwich, while modules 2 and 4 constitute a discontinuous β-barrel fold. The segregation into these two subdomains becomes even more

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2 The names of the chimeras illustrate the composition from modules (the subscript numbers) of TBG and α1PI (preceding letter T or P, e.g. in P1T2P3−4, modules 1, 3, and 4 are α1PI sequences, whereas the second module is a TBG sequence. The denotation of serpin secondary structure elements and their assignments to TBG are as described in Ref. 17.
proteins. The corresponding hybrid cDNAs, generated by repeated cycles of splicing by overlap extension polymerase chain reaction, were used to produce recombinant baculovirus by in vivo recombination in insect cells.

Expression of Recombinant Proteins and Reaction with HLE—Bv-α1PI, bv-TBG, and the three chimeras were efficiently secreted by the insect cells with similar expression levels of up to 5 μg/ml after 60 h in serum-free medium. The structural integrity of the proteins was evident by their reaction with specific polyclonal anti-α1PI and anti-TBG antibodies, whereas there was no detectable cross-reactivity between bv-TBG, bv-α1PI, or wild type baculovirus with these antisera.

Chimera P1T2P3–4 and, to a lesser extent, P1T2P3T4 retained the inhibitory properties of α1PI and formed SDS-stable complexes with HLE (Fig. 3). P1T2P3–4 and P1T2P3T4 showed significantly more cleaved inhibitor than bv-α1PI. The reaction of HLE with P1T2P3–4 was slower than with α1PI, as indicated by the large amount of uncleaved inhibitor at a molar ratio of one. In contrast to the stable reaction products of cleaved bv-α1PI, increasing HLE concentrations led to a loss of detectable P1T2P3–4-HLE complex concomitant with the disappearance of free P1T2P3–4 (Fig. 4). As expected, bv-TBG and P1T2–4 harboring the RSL equivalent of TBG behaved like pure substrates (Fig. 3).

Inhibitor Assay—The residual proteolytic activity of HLE preincubated with increasing amounts of the inhibitors showed a linear dependence characteristic for tight binding inhibition (Fig. 5). The stoichiometries of inhibition (SI), defined as mole of serpin required to inhibit 1 mole of HLE, were 1.3 for bv-α1PI, 2.1 for P1T2P3–4, and 11 for P1T2P3T4. These SI values were in agreement with the reaction products on the immunoblots (Figs. 3 and 4). Again, bv-TBG and P1T2–4 showed no inhibition of HLE.

Analysis of T4 Binding—Scatchard analysis of T4 binding showed no detectable T4 binding activity (Kd < 10⁶ M⁻¹) for chimera P1T2P3–4 and the bv-α1PI control. However, in P1T2P3T4 transposition of the complete β-barrel motif into the α1PI frame created a high affinity T4 binding site (Kd = 1.7·10⁸ M⁻¹), comparable with the first binding site of transthyretin (Fig. 6B). The additional substitution of module 3 in P1T2–4 increased the T4 binding affinity to almost half of that of bv-TBG or human serum TBG (Kd = 0.5·10⁻¹⁰ M⁻¹ versus 1.2·10⁻¹⁰

Fig. 3. Immunoblot of chimeras before and after incubation with HLE. Bv-α1PI, bv-TBG, and the chimeras were incubated either alone (–) or with (+) an equimolar amount of HLE for 20 min at 37 °C. Nondigested and digested samples were separated on nonreducing SDS-PAGE, blotted, and probed with polyclonal anti-α1PI (upper panel) and anti-TBG antibodies (lower panel). P1T2P3–4 formed SDS-stable complexes with HLE similar to bv-α1PI but also showed a significant substrate reaction. Chimera P1T2P3T4 also formed an HLE-inhibitor complex (detected with both antibodies), but most of the protein was cleaved. P1T2–4 and bv-TBG showed pure substrate reactions.
The fraction of complexes P1T2P3T4 was smaller and less stable than that of bv-α1PI.

To examine the effect of the binding affinity of P1T2P3T4 (●) was 70 times less than bv-TBG, but at 1.7 × 10^{-10} M\(^{-1}\) it was still higher than the second-best natural T\(_4\)-binding protein, transthyretin (○) (K\(_d\) = 9.9 × 10^{-10} M\(^{-1}\)). α1PI and P1T2P3T4 had no specific T\(_4\) binding. The plots are representative of four independent experiments.

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**Fig. 5.** Titration of HLE with α1PI-TBG chimeras. HLE was titrated with each of the chimeras at 37 °C. After incubation for 15 min, residual HLE activity was determined. For bv-α1PI, P1T2P3T4, and P1T2P3, linear titration curves were obtained irrespective of the substrate concentrations tested (0.1 and 1 mM, K\(_{d}\) of 0.15 mM). The intercept with the abscissa yielded an apparent stoichiometry of 1.3 for bv-α1PI (○), 2.1 for P1T2P3 (●), and 11 for P1T2P3T4 (○). Bv-TBG (□) and P1T2–4 (△) showed no inhibition of HLE even at a high molar excess.

**Fig. 6.** T\(_4\) binding of α1PI-TBG chimeras. A, Scatchard analysis of T\(_4\) binding of bv-TBG (□) and human serum TBG revealed no significant differences in binding affinity (K\(_d\) = 1.2 ± 0.11 × 10^{-10} M\(^{-1}\)). The K\(_d\) of chimera P1T2P3T4 (△) was only slightly reduced (0.5 ± 0.14 × 10^{-10} M\(^{-1}\)). B, the binding affinity of P1T2P3T4 (●) was 70 times less than bv-TBG, but at 1.7 × 10^{-10} M\(^{-1}\) it was still higher than the second-best natural T\(_4\)-binding protein, transthyretin (○) (K\(_d\) = 9.9 × 10^{-10} M\(^{-1}\)). α1PI and P1T2P3T4 had no specific T\(_4\) binding. The plots are representative of four independent experiments.

**Fig. 7.** Native PAGE analysis of heat-denatured chimeras. Samples were incubated with increasing ratios of HLE (E/I). Reactions were stopped after 20 min by denaturation at 95 °C in 0.1% SDS. The fraction of complexes P1T2P3T4 was smaller and less stable than that of bv-α1PI.

**Fig. 8.** Functional stability of chimeras. A, rate of thermal inactivation for P1T2P3T4, P1T2–4, and bv-TBG as determined by residual T\(_4\) binding activity was determined. Values are expressed as protein-bound T\(_4\) relative to the basal levels and represent the means ± SD for three independent experiments. Plots of the log binding capacities versus time of incubation were linear, indicative of an apparent first-order process. Bv-TBG (□) had a slightly reduced functional stability compared with human serum TBG (○) (t\(_{1/2}\) of 4.5 versus 7 min), whereas P1T2P3T4 (△) was rapidly denatured (t\(_{1/2}\) = 2 min). Note that P1T2P3T4 (●) is essentially stable at 60 °C with no significant loss of T\(_4\) binding capacity within 30 min. B, heat denaturation profile illustrating the markedly increased functional stability of uncleaved P1T2P3T4 comparable with bv-TBG cleaved by HLE (x). C, functional stability measured by means of the residual HLE inhibitory activity. P1T2P3T4 lost its inhibitory potency at temperatures slightly lower than bv-α1PI (○), whereas P1T2P3 (●) was less stable in this assay.

Consistent with the increased conformational stability on native PAGE, P1T2P3T4 displayed no significant decline of T\(_4\) binding after incubation at temperatures as high as 85 °C for 20 min (Fig. 8B). However, its inhibitor function was lost at a slightly lower temperature than that of bv-α1PI, starting at 55 °C (Fig. 8C). SDS-PAGE analysis of P1T2P3T4 denatured at 65 °C revealed that this material was still a specific substrate...
for HLE but did not form a serpin-enzyme complex (data not shown).

The inhibitor function of chimera P1T2P3–4 was also less stable than that of bv-αPI and was completely inactivated at 55 °C. The $t_{1/2}$ (60 °C) of T4 binding of P1T2–4 was reduced to about one-third of the $t_{1/2}$ of bv-TBG (Fig. 8A).

**DISCUSSION**

Genetic engineering has become a mainstay in elucidating the still inadequately understood structure-function correlation of proteins. This information is critical for the understanding of the diversity of proteins and the design of new drugs. In recent years, research has moved from the substitution of single amino acids to the concept of a modular design of proteins. In some proteins, structural and functional units are readily obvious, e.g., the extra- and intracellular and the transmembrane domains of membrane-bound receptors. The identification of discrete units has been used for the successful construction of chimeric receptors (37). However, in chimeras of globular proteins so far only similar functions have been substituted (38–41). In this study, we present a strategy to engineer bifunctional chimeras from integral parts of homologous proteins. Based on a concept of a modular architecture of the serpins (Fig. 9), we have combined two different functional properties of the serpin superfamily, proteinase inhibition and ligand binding, into one chimeric molecule. The inhibitory and ligand-binding characteristics of the chimeras are summarized in Table II.

In chimera P1T2P3T4, the transfer of the T4-binding site of TBG into the αPI frame was achieved by substituting the β-barrel-like structure of αPI with its TBG homologue (modules 2 and 4). This chimera exhibited inhibition of and complex formation with HLE, characteristic of inhibitory serpins such as αPI. In comparison with the archetypical, evolutionarily refined αPI, it was a weaker inhibitor with a higher apparent stoichiometry of inhibition and a shorter half-life of its complex. In addition to proteinase inhibition, chimera P1T2P3T4 also exhibited a specific, high affinity T4 binding. Although its binding affinity was 70-fold lower than that of TBG, it was still higher than that of transthyretin, the next best natural T4 binding protein.

In contrast, the substitution of only module 2 and thus only part of the αPI β-barrel including the environment of the affinity-labeled Lys253 (24) did not result in detectable T4 binding. Similarly, a chimera harboring only module 4 of TBG produced a dysfunctional, secretion-deficient protein (data not shown). Only the substitution of the complete β-barrel, comprising modules 2 and 4, was sufficient to transfer the high affinity T4-binding site. Consequently, both modules seem to participate in avid T4 binding, in agreement with the demonstration that all parts of the T4 molecule, and thus an extensive surface of interaction of T4 with the binding cavity of TBG, are essential for its high binding affinity (42). Furthermore, the functional transfer of the T4-binding site of TBG into the αPI frame unambiguously locates the ligand-binding site to the β-barrel motif of the serpins.

Surprisingly, P1T2P3T4 remained in solution and retained its T4 binding activity even at remarkably high temperatures (Fig. 8). Serpins tend to polymerize at elevated temperatures (43) and simultaneously lose their activity and escape immunodetection as a result of precipitation. Polymerization is thought to involve the insertion of the loop of one serpin molecule into either sheet A (44) or C (21, 45) of another molecule. Both models require detachment of strand 1 from the C-sheet (46). The extended RSL of chimera P1T2P3T4, which is engineered to be 3 or 7 amino acids longer than in TBG or αPI, respectively (Fig. 10), most likely delays the heat-induced release of strand 1C from the C-sheet, compatible with the increased thermal resistance of an αPI variant with a C-terminal extended RSL (48).

The discrepancy in the functional stability of T4 binding versus HLE inhibition of P1T2P3T4 could be the result of a higher intrinsic stability of the β-barrel than the remaining molecule. Significant heat-induced unfolding might occur without affecting the β-barrel and thus T4 binding. However, the cooperativity in the unfolding of serpins (19, 49) argues against this possibility. More conceivably, a local structural rearrangement of the RSL is responsible for the observed loss of inhibitory activity at intermediate temperatures. During heat exposure the A-sheet of the serpins is supposed to open up and accept a portion of its own RSL. In P1T2P3T4 this might distort the RSL near the scissile bond, resulting in a pure substrate behavior toward HLE, whereas the extension of the RSL prevents detachment of s1C and hence both polymerization and loss of T4 binding. This limited structural transition of P1T2P3T4 might resemble the spontaneous conversion of plasminogen to plasmin.

| SI  | $K_a$ | $10^{15}$ M$^{-1}$ | $10^{16}$ M$^{-1}$ |
|-----|-------|------------------|------------------|
| bv-TBG | 1.3 | 0.35 | 0.56 |
| P1T2 | 1.2 | 0.37 | 0.61 |
| P1T2P3T4 | 11 | 0.21 | 0.36 |
| P1T2P3T4 | 2.1 | 0.013 | 0.022 |
| bv-αPI | 1.3 | No detectable inhibition. |

* No detectable inhibition.

**Fig. 9.** The proposed modular architecture of the serpins. Ribbon drawing of α1PI with the proposed compact modules depicted in different colors. Module 1 (Phe229–Met223) is shown in pink, module 2 (Lys222–Leu288) in blue, module 3 (Pro299–Pro315) in yellow, and module 4 (Pro326–Lys394) in green. The reactive center residues within the RSL are highlighted as space-filling models. Coordinates were taken from Protein Data Bank entry 1PSI (34).
minogen activator inhibitor-1 from an active to a latent conformation in vivo (50, 51).

In conclusion, the successful construction of a bifunctional chimera clearly demonstrates that ligand binding and proteinase inhibition are not exclusive within the serpin structure and provides evidence for their proposed modular architecture. Moreover, because our approach does not rely on specific features of the serpins but rather uses general design criteria such as compactness of modules and sequence conservation at fusion points, it appears not to be limited to this protein superfamily. There are many examples in which unrelated functions have evolved within a conserved structural scaffold (52–54), occasionally recruiting different portions of a molecule as reactive centers (55, 56). Thus the exchange of homologous modules offers vast possibilities for the design of chimeric proteins with new functional properties. Furthermore, the integration of two functions in one globular protein suggests the potential to introduce novel allosteric effects, e.g. modulation of enzymatic activities upon ligand binding.

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