Binding Kinetics, Structure-Activity Relationship, and Biotransformation of the Complement Inhibitor Compstatin[1]

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We have previously identified a 13-residue cyclic peptide, Compstatin, that binds to complement component C3 and inhibits complement activation. Herein, we describe the binding kinetics, structure-activity relationship, and biotransformation of Compstatin. Biomolecular interaction analysis using surface-plasmon resonance showed that Compstatin bound to native C3 and its fragments C3b and C3c, but not C3d. While binding of Compstatin to native C3 was biphasic, binding to C3b and C3c followed the 1:1 Langmuir binding model; the affinities of Compstatin for C3b and C3c were 22- and 74-fold lower, respectively, than that of native C3. Analysis of Compstatin analogs synthesized for structure-function studies indicated that 1) the 11-membered ring between disulfide-linked Cys[2]-Cys[12] constitutes a minimal structure required for optimal activity; 2) retro-inverso isomerization results in loss of inhibitory activity; and 3) some residues of the type I β-turn segment also interact with C3. In vitro studies of Compstatin in human blood indicated that a major pathway of biotransformation was the removal of Ile[1], which could be blocked by *N*-acetylation of the peptide. These findings indicate that acetylated Compstatin is stable against enzymatic degradation and that the type I β-turn segment is not only critical for preservation of the conformational stability, but also involved in intermolecular recognition. ***The Journal of Immunology,*** **2000, 165: 2491–2499.**

Complement component C3[4] is a common denominator in the activation of the classical, alternative, and lectin pathways of complement activation. Proteolytic activation of C3 by classical/lectin (C4b, 2a) or alternative (C3b, Bb) pathway C3 convertases results in covalent attachment of this molecule to the target cells. C3b deposition leads to enhanced phagocytosis, increased Ab production, generation of C5a, and formation of the C5b-9 membrane attack complex on target cells.

Complement activation is essential for the development of normal inflammatory responses against foreign pathogens; however, its inappropriate activation has been a cause of tissue injury in many disease states. This tissue injury is directly mediated by the membrane attack complex, C5b-C9, and indirectly by the generation of anaphylatoxic peptides C3a, C4a, and C5a. Complement-mediated tissue injury has been reported in a variety of disease states, including autoimmune diseases (1), acute respiratory distress syndrome (2), Alzheimer's disease (3, 4), stroke (5), heart attack (6), burn injuries (7), and reperfusion injuries (8, 9). Complement-mediated tissue injury has also been found to occur as a consequence of bioincompatibility situations, such as those encountered during dialysis and cardiopulmonary bypass (10, 11) and xenotransplantation (12, 13). Thus, there is a clear need for specific complement inhibitors.

To identify specific small-m.w. complement inhibitors that block all three pathways of complement activation, we previously screened a phage-displayed random peptide library with C3b, a proteolytically activated form of complement C3. This approach led to the identification of a 13-residue cyclic peptide (14), later named Compstatin. Studies of the mechanism of complement inhibition by Compstatin revealed that unlike natural inhibitors of complement that act on C3b, Compstatin binds to native C3 and inhibits its cleavage by C3 convertase (14); this inhibition was not caused by sterically hindered access to the C3a/C3b cleavage site (14). Three-dimensional (3D) structural analysis of a major conformer of Compstatin in solution, using two-dimensional (2D) nuclear magnetic resonance (NMR) and a hybrid distance geometry-restrained simulated annealing methodology, revealed that the backbone forms a type I β-turn comprising residues Gln[5]-Asp[6]-Trp[7]-Gly[8] (15). It is noteworthy that analyses of Ala substitution analogs indicated that in addition to Val[3], type I β-turn residues (Gln[5]-Gly[8]) contribute significantly to the inhibitory activity of the peptide (15).

Thus far, Compstatin has been tested in three different clinically relevant models. Hyperacute rejection in discordant kidney xenotransplantation has been studied ex vivo using a porcine-to-human perfusion model. In this model, Compstatin significantly prolonged the survival of the kidneys (12, 13). Its effect has also been tested in models for extracorporeal circulation (16), where it effectively inhibited the generation of C3a and sC5b-9 and the binding of C3/C3 fragments to a polymer surface. As a result of the

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[4] Abbreviations used in this paper: C3, third component of complement; C3(H_2O), C3 with a hydrolyzed thioester bond; C3b, the proteolytically activated form of C3; C3c, the 135,300 M_r fragment of C3 generated using elastase; C3d, the 35,000 M_r fragment of C3 generated using elastase. Er, rabbit erythrocytes; NMR, nuclear magnetic resonance; VBS, Veronal-buffered saline; GVB, VBS containing 0.1% gelatin; SPR, surface plasmon resonance; RU, response unit; NHS, normal human serum; 3D, three-dimensional; 2D, two-dimensional; TFA, trifluoroacetic acid; Ac-Compstatin, acetylated Compstatin.

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inhibition of complement activation, the activation of polymorphonuclear leukocytes (assessed by the expression of CD11b) and the binding of these cells ($CD16^+$) to the polymer surface were almost completely lost (16). Most recently, Compstatin has been tested in vivo in primates to examine its effect on complement activation induced by a heparin-protamine complex; here it effectively inhibited complement activation (17).

This study was designed to characterize the interaction of Compstatin with C3. Here, using surface plasmon resonance (SPR), we have compared the interaction of Compstatin with native C3, C3(H_2O) (C3 with a hydrolyzed thioester bond), and with the C3 fragments C3b, C3c, and C3d. The data we obtained point to differences in the mechanism of binding among these interactions. In addition, data from our structure-function analysis of Compstatin suggest that residues involved in type I β-turn formation are also important for binding to C3. We have also performed biotransformation studies of this peptide to evaluate its stability in human blood; these findings indicate that Compstatin is stable in human blood and that *N*-acetylation of this peptide enhances its stability against plasma proteases.

Materials and Methods

Chemicals and buffers

All chemicals and reagents used for peptide synthesis were purchased from Applied Biosystems (Foster City, CA), with the exception of F-moc amino acids, which were obtained from Nova Biochem (San Diego, CA). Veronal-buffered saline (VBS), pH 7.4, contained 5 mM barbital and 145 mM NaCl. Gelatin VBS (GVB) was VBS containing 0.1% gelatin; GVB^{2+} was GVB containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$; and GVBE was GVB with 10 mM EDTA. MgEGTA contained 0.1 M $MgCl_2$ and 0.1 M EGTA. PBS contained 10 mM phosphate, 145 mM NaCl, pH 7.4. PBS-T was PBS containing 0.05% Tween 20.

Purified proteins

Human complement protein C3 was purified from normal human plasma as described (18). To separate native C3 from C3(H_2O), the C3 sample was incubated at 37°C for 2 h, loaded onto a Mono S column (Pharmacia, Piscataway, NJ), and eluted with a linear salt gradient (19). C3b was generated by limited trypsin cleavage of C3 and purified on a Mono Q column (Pharmacia) (20). C3c and C3d were generated by elastase treatment of C3 and purified on a Mono Q column (Pharmacia) (20).

Peptide synthesis, purification, and characterization

Compstatin and its analogs were synthesized in an Applied Biosystems peptide synthesizer (model 431A) using F-moc amide resin (4-(2′,4′-dimethoxyphenyl-F-moc-aminomethyl)-phenoxy resin). The side-chain protecting groups were Cys (Trt), Asp (otBu), Arg (Pmc), Thr (tBu), Gln (Trt), Trp (Boc), and His (Trt). All peptides were cleaved from the resin by incubation for 3 h at 22°C with a solvent mixture containing 5% phenol, 5% thioanisole, 5% water, 2.5% ethanedithiol, and 82.5% trifluoroacetic acid (TFA). The reaction mixture was filtered through a fritted funnel, precipitated with cold ether, dissolved in 50% acetonitrile containing 0.1% TFA, and lyophilized. The crude peptides obtained after cleavage were dissolved in 10% acetonitrile containing 0.1% TFA and purified using a reversed-phase C-18 column (Waters, Milford, MA). Disulfide oxidation of peptides VII-XV was performed by stirring a 0.15-mM solution of the peptide in 0.1 M ammonium bicarbonate, pH 8.0, and bubbling with oxygen at 22°C for 48 h. Disulfide oxidation of all the other peptides was done on-resin as described elsewhere (13). Purified peptide II was reduced and alkylated with 10 mM DTT and 40 mM iodoacetamide. *N*-Acetylation of the peptides was achieved by treating them with a solution of 0.5 M acetic anhydride, 0.125 M diisopropylethylamine, and 0.015 M 1-hydroxybenzotriazide for 5 min (Applied Biosystems User Bulletin no. 35). On-resin biotylation of the peptides was performed as follows. The Dde group on the Lys side chain was removed by treating the peptide-resin with three short treatments (3 min each) of 2% hydrazine in dimethylformamide (21). It was then placed on the synthesizer and washed with *N*-methylpyrrolidone. Biotin (1 mmol) was dissolved in equal volumes (2.9) of DMSO and *N*-methylpyrrolidone, placed in an amino acid cartridge, and activated according to Applied Biosystems User Bulletin no. 35. The purity and identity of Compstatin and its analogs were critically monitored by analytical chromatography on a reversed-phase C-18 column and by laser desorption mass spectrometry (22). Formation of a disulfide bond in each cyclic peptide was confirmed by mass spectrometry using a mass shift assay that involves reaction of thiols with *p*-hydroxy mercuribenzoic acid (23).

SPR measurements

The kinetics of Compstatin binding to C3 and its fragments were determined on SPR-based biosensors Biacore X and Biacore 3000 (Biacore, Piscataway, NJ) at 25°C. Binding experiments were performed in physiologic ionic strength buffer (PBS-T) using a streptavidin chip (Sensor Chip SA; Biacore). Nonspecific adsorption of analytes to the sensor chip was blocked by adding 0.05% Tween 20 to the buffer. To obtain a homogeneous ligand surface, Compstatin analogs were oriented on the sensor chip. Approximately 100–120 response units (RUs) of biotinylated Compstatin (I∗CVVQDWGHHRC∗TAGHMANLTSHASAK-biotin or biotin-KYSSI∗CVVQDWGHHRC∗T-NH_2) was immobilized on the test flow cell, and the control flow cell was immobilized with equivalent RUs of the linear analog (IAVVQDWGHHRATAGHMANLTSHASAK-biotin). Addition of a spacer (extra residues) at the N or C terminus was based on the sequence of the parent phage peptide. Binding was measured at 30 μl/min by injecting various concentrations of analyte for 120 s; dissociation was followed for an additional 120 s. The sensor chip was regenerated by brief pulses of 0.2 M sodium carbonate, pH 9.5. Biosensor data for the control analog were subtracted from those obtained for biotinylated Compstatin and globally fit to a 1:1 Langmuir binding model:

$$A + B \rightleftharpoons AB \tag{1}$$

using BIAevaluation 3.0 (Biacore). Because the biosensor data for native C3 and C3(H_2O) did not fit to a simple 1:1 model (the derivatives of the binding response and the dissociation were nonlinear), the binding data were evaluated by linear transformation analysis as previously described (24, 25). For a simple bimolecular interaction (Equation 1), the association and dissociation processes are described, respectively, as:

$$dRU/dt = k_{on}[A]RU_{max} - (k_{on}[A] + k_{off})\,RU \tag{2}$$

and

$$RU = RU_0 e - k_{off}t \tag{3}$$

In these equations, k_{on} and k_{off} are the association and dissociation rate constants, respectively; [*A*] is the concentration of analyte injected, and *B* is the immobilized ligand; *RU* is the relative response of the biosensor at time *t* and is proportional to the amount of complex formed; RU_{max} is the maximum response; and RU_0 is the response at the beginning of the dissociation. Linear analysis of the binding data uses the slope (k_s) of the line obtained by plotting *dRU*/*dt* vs *RU* to determine the association rate constant (k_{on}), where $k_s = -(k_{on}[A] + k_{off})$. A secondary plot of k_s vs [*A*] will have a slope of k_{on}. For a single exponential process (Equation 3), a plot of $\ln(RU_0/RU)$ vs time will yield a straight line with a slope of k_{off}. The equilibrium dissociation constant (K_D) is calculated from the equation $K_D = k_{off}/k_{on}$.

Complement-mediated hemolytic assays

Inhibition of complement activity by Compstatin and its peptide analogs was studied by measuring their effect on the alternative pathway of the complement. Inhibition of complement activation was determined by measuring the lysis of rabbit erythrocytes (Er) in normal human serum (NHS) as previously described (26). Various concentrations of peptides were mixed with 5 μl of NHS, 5 μl of 0.1 M MgEGTA, and 10 μl of Er (1×10^9/ml), and the final volume was adjusted to 100 μl with GVB. The reaction mixture was incubated at 37°C for 20 min and stopped by adding 200 μl of GVB with 10 mM EDTA. After centrifugation, lysis of Er was determined at 405 nm. The percentage of lysis obtained was normalized by considering 100% lysis to be equal to the lysis occurring in the absence of the peptide. The concentration of the peptide causing 50% inhibition of hemolytic activity was taken as the IC_{50}.

In vitro biotransformation

One milliliter of fresh heparinized human blood or NHS was mixed with 0.3 mg of Compstatin or one of its analogs (dissolved in 1 ml of PBS) and incubated at 22°C. Samples (200 μl) were removed at various time intervals and immediately centrifuged at 4°C to separate plasma from cells. One hundred microliters of plasma was removed, mixed with an equal volume of 0.1% TFA in H_2O, and centrifuge-filtered through Ultrafree-MC filters (Millipore, Bedford, MA) with a molecular mass cut-off of 5000 Da. Half of the filtrate was injected onto HPLC (Waters LC 600) connected to a reverse-phase C-18 column (Vydac, Heperia, CA), and the other half was

analyzed by matrix-assisted laser desorption mass spectrometry using a time-of-flight mass spectrometer (MicroMass TofSpec, Beverly, MA). Each peak recovered from HPLC was also individually characterized by matrix-assisted laser desorption mass spectrometry (22) and Edman degradation. The first sample, removed immediately after starting the incubation, was considered the zero time point.

Results

Binding kinetics of the Compstatin-C3 interaction

To measure the affinity of Compstatin for C3, and to better understand the nature of the Compstatin-C3 interaction, we used SPR technology. In general, immobilization of the ligand is achieved by amine-coupling chemistry in this technology; however, this approach produces heterogeneous surface ligands and precludes the measurement of homogeneous binding constants. Thus, we synthesized Compstatin analogs with biotin added to its N or C terminus and oriented these analogs onto a chip surface. Both of these analogs (peptides XXII and XXIII) were as active as Compstatin in inhibiting complement activity (Table I). A linear analog of Compstatin (peptide XXIV) was used as a control, because it did not bind to C3. Compstatin analog oriented via its C terminus (peptide XXIII) bound to C3 (Fig. 1), whereas the analog oriented via its N terminus (peptide XXII) failed to bind to C3 (data not shown), suggesting that a free N terminus is important for peptide binding to C3. It should be pointed out that Compstatin, when originally isolated from a phage-displayed random peptide library, was oriented in the M13 phage via its C terminus.

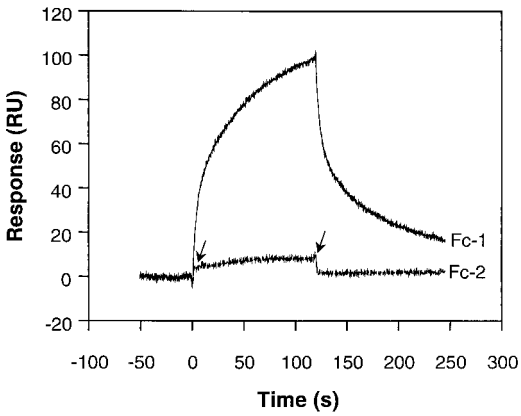

FIGURE 1. Raw sensograms showing binding of Compstatin to C3. A solution of C3 (270 μM) was injected over a streptavidin sensor chip containing a biotinylated analog (peptide XXIII) immobilized on flow cell-1 (Fc-1) and a linear analog (control peptide XXIV) immobilized on flow cell-2 (Fc-2). Arrows indicate the bulk change in refractive index due to the difference in solution composition between the running buffer and the protein solution.

The binding data (sensograms) for Compstatin-C3 interaction could not be fitted to a simple 1:1 binding model. Linear transformation of the association and dissociation data according to Equations 2 and 3, respectively, showed nonlinear plots. Nonlinearity in

Table I. *Amino acid sequences, mass spectral analyses, and complement inhibitory activities of Compstatin and its analogs*

Peptide No.	Amino Acid Sequence[a]	Mass Spectral Analyses: Expected	Mass Spectral Analyses: Observed	Inhibition of Human Complement[b] IC_{50} (μM)	Biotransformation
Compstatin and linear analogs					
I	I*CVVQDWGHHRC*T-NH_2 (Compstatin)	1552	1551	12	+[c]
II	ICVVQDWGHHRCT-NH_2[d] (linear)	1660	1664	>600[e]	ND
III	IAVVQDWGHHRAT-NH_2 (linear)	1489	1492	>600	+
IV	*CVVQDWGHHR..C*T-NH_2[f] (linear)	1458	1456	>300	ND
V	Ac-I*CVVQDWGHHRC*T-NH_2	1593	1593	4	–
Deletion analogs					
VI	*CVVQDWGHHRC*T-NH_2	1438	1439	25	–
VII	*CVVQDWGHHRC*-NH_2	1337	1337	33	–
VIII	*CHHRC*T-NH_2	753	755	>600	–
IX	*CGHHRC*T-NH_2	810	813	>600	–
X	*CWGHHRC*T-NH_2	996	997	>600	–
XI	*CDWGHHRC*T-NH_2	1111	1113	>600	–
XII	*CQDWGHHRC*T-NH_2	1239	1240	>600	–
XIII	*CVQDWGHHRC*T-NH_2	1339	1339	>600	–
XIV	*CVVQDWC*-NH_2	849	851	>600	–
XV	*CVVQDWGHC*-NH_2	1043	1047	>600	–
Retro-inverso analog					
XVI	*dCdRdHdHdGdWdDdQdVdVdC*	1337	1338	>500	–
β-turn analogs					
XVII	Ac-I*CVVGPFGHHRC*T-NH_2	1465	1464	>400	–
XVIII	Ac-I*CVVGDWGHHRC*T-NH_2	1522	1522	567	–
XIX	Ac-I*CVVQPWGHHRC*T-NH_2	1575	1577	98	–
XX	Ac-I*CVVQDFGHHRC*T-NH_2	1554	1554	>400	–
XXI	Ac-I*CVVGPdFGHHRC*T-NH_2	1467	1467	>150	–
Biotinylated analogs					
XXII	Biotin-KYSSI*CVVQDWGHHRC*T-NH_2	2243	2241	3	ND
XXIII	Ac-I*CVVQDWGHHRC*TAGHMANLTSHASAK-Biotin	3196	3195	4	ND
XXIV	Ac-IAVVQDWGHHRATAGHMANLTSHASAK-Biotin	3134	3134	>300	ND

[a] Asterisks denote oxidized cysteines.
[b] Complement activities were determined by measuring alternative pathway-mediated lysis of Er.
[c] Only limited first cleavage (i.e., removal of Ile^1) was seen under these conditions.
[d] Peptide II was reduced with DTT and alkylated using iodoacetamide.
[e] Maximum concentration of peptide that could be used in the assay because of solubility limitations.
[f] Cleaved between Arg and Cys.

the binding data could have been due to the presence of C3(H_2O) in the sample, because freeze-thawing (27) and spontaneous hydrolysis of the thioester bond convert C3 into C3(H_2O) (28). To test this possibility, we separated native C3 from C3(H_2O) on a Mono S column (Pharmacia) (19). C3(H_2O) was generated in bulk by repeated cycles of freezing and thawing and then purification over a Mono S column (19). When purified native C3 and C3(H_2O) were tested for binding to Compstatin, both bound in a nonlinear fashion (Fig. 2), indicating that binding follows complex models. Thus, the *dR*/*dt* plots were divided into fast and slow components. Such approximations have been used previously (24, 25). The k_s values for the fast and slow components obtained from the slopes of the *dR*/*dt* plots (Equation 2) were plotted against C3 concentrations to calculate k_{on} values (Fig. 2, *insets*, and Table II).

To calculate dissociation constants, the dissociation phase of the highest concentration was replotted as ln (response at time zero of dissociation/response at time *n*) (Fig. 3). Dissociation is typically measured using the highest concentration, because doing so yields the most reproducible data. The dissociation rate constant was calculated from the slope of the line defined by the initial data points of this plot. Analysis of the data indicated that native C3 and C3(H_2O) bound to Compstatin with nearly identical affinity (Table II).

To localize the region of C3 involved in the interaction with Compstatin, binding experiments were also performed using the C3 fragments C3b, C3c, and C3d. Compstatin bound to C3b and C3c but failed to bind to C3d, indicating that the binding site resides in the C3c region of C3 (Fig. 2 and Table II). The kinetics of binding of C3b and C3c were distinctly different from those of native C3 and C3(H_2O). The sensograms for C3b and C3c, except during the later part of the dissociation, showed a close fit to a 1:1 binding model. We reasoned that the poor fit during the later part of the dissociation might be due to rebinding of the analyte molecules. To verify this possibility, we coinjected 110 μM Compstatin during the dissociation phase. As shown in Fig. 4, injection of the free peptide during the dissociation of C3b decreased the residual signal during the later part of the dissociation and yielded a close fit to a 1:1 binding model; similar results were obtained when the free peptide was injected during the dissociation of C3c (data not shown). Both C3b and C3c showed a decrease in their on-rates when compared with C3; little change was observed in the off-rates. This decrease in on-rates resulted in 22- and 74-fold decreases in affinity for C3b and C3c, respectively.

Identification of the structural features of Compstatin that are critical for its complement inhibitory activity

In an earlier study (14) we showed that Compstatin, the cyclic 13-aa N-terminal region of the parent phage-displayed peptide, effectively inhibits human complement activity. In this study, we have tested the activity of Compstatin and its several analogs by measuring their ability to inhibit the alternative pathway-mediated lysis of Er (Fig. 5). We interpreted the loss of activity in some of these analogs to their inability to bind to C3. Hemolytic assays use serum as a source of complement; therefore, a loss or reduction in activity could result from proteolytic cleavage of the Compstatin peptide in serum. To conclusively rule out this possibility, we checked for peptide cleavage under the same conditions. None of the analogs tested, with the exception of peptides I and III, were cleaved under these conditions (Table I). Peptide I showed limited loss of Ile^1, and peptide III showed limited cleavage between Ile^1-Ala^2, Ala^2-Val^3, and Val^3-Val^4.

To localize the minimal functional region of Compstatin, we made a series of deletion analogs. Compstatin contains two flanking amino acid residues outside the constrained region. Deletion of both of these residues or of the N-terminal residue (Ile^1) resulted in a 2-fold reduction in activity, indicating that Ile^1 enhances the inhibitory activity of the peptide (peptides VI and VII, Table I). To further reduce the size of the peptide, we generated short constrained peptides by changing the ring size; one to six residues inside the 11-member ring were deleted (peptides VIII-XV, Table I). However, we found that we were unable to further reduce the size of the Compstatin peptide and still retain activity. Identification of the residues of Compstatin that are involved in the interaction with C3 and elucidation of the 3D structure of these analogs, if amenable to NMR analysis, are needed to interpret these results. Nevertheless, these findings indicate that the 11-member peptide between disulfide-linked Cys^2 and Cys^{12} constitutes a minimum structure required for optimal activity.

Protease-resistant peptide mimetics synthesized entirely from D-amino acids in the reverse order of their all-L-homologs (retro-inverso) are known to retain biological activity (29). In such peptides, the side-chain configurations correspond to the natural all-L-form derivative, but the carbonyl and amine groups forming the backbone amide bonds are reversed (30). Therefore, retro-inverso peptides are considered as useful probes for dissecting the relative contribution of side-chain and main-chain interactions to a molecular recognition process. Therefore, we synthesized the retro-inverso mimetic (peptide XVI, Table I) of Compstatin and tested its activity. It was found to be inactive, suggesting that main-chain atoms of Compstatin contribute to C3 recognition.

Our previous data on Ala scan analogs indicated that Val^3, Gln^5, Asp^6, Trp^7, and Gly^8 are important for maintaining the inhibitory activity of Compstatin (15). From our 2D NMR studies, it was clear that Val^3 participates in side-chain hydrophobic interactions and that Gln^5-Gly^8 form a type I β-turn, which is important for the structural stability of Compstatin and probably for specific side-chain orientation. However, it was not clear whether type I β-turn residues are also involved in the interaction with C3. To dissect the role of these residues in the structural stability of Compstatin, as contrasted with its interaction with C3, we synthesized β-turn analogs and analyzed their activity. The residues Gln^5-Asp^6-Trp^7-Gly^8, which form the type I β-turn in Compstatin, were substituted either with Gly-Pro-Phe-Gly (peptide XVII) or with Gly-Pro-DPhe-Gly (peptide XXI), which have the propensity to form type I and type II β-turns, respectively (31). Both analogs were inactive (Table I), suggesting that in addition to providing structural stability, some of the β-turn residues must also interact with C3. Substitution of individual turn residues with residues typical of those positions in a type I β-turn (i.e., Gln^5-Gly, Asp^6-Pro, and Trp^7-Phe substitutions, peptides XVIII-XX) resulted in a substantial decrease in the inhibitory activity of the peptide. The maximal loss (>100-fold) in activity was observed with the Gln^5-Gly and Trp^7-Phe substitutions, suggesting that these residues might be involved in intermolecular recognition. Analysis of complete sets of 2D NMR experiments for a number of analogs with substituted β-turn residues is underway to dissect the role of the turn in binding and inhibitory activity of Compstatin.

In vitro biotransformation of Compstatin in human blood

The properties of Compstatin (13, 16) suggest that it has therapeutic usefulness as an anticomplement drug; thus, it is important to determine its biochemical and functional stability in human blood. Compstatin was incubated in human blood, and its biotransformation products were separated by HPLC on a reversed-phase C-18 column and characterized by matrix-assisted laser desorption-ionization mass spectrometry and Edman degradation. The membrane filtration method used in this study recovered about 70% of the peptide added to the blood. Incubation of Compstatin

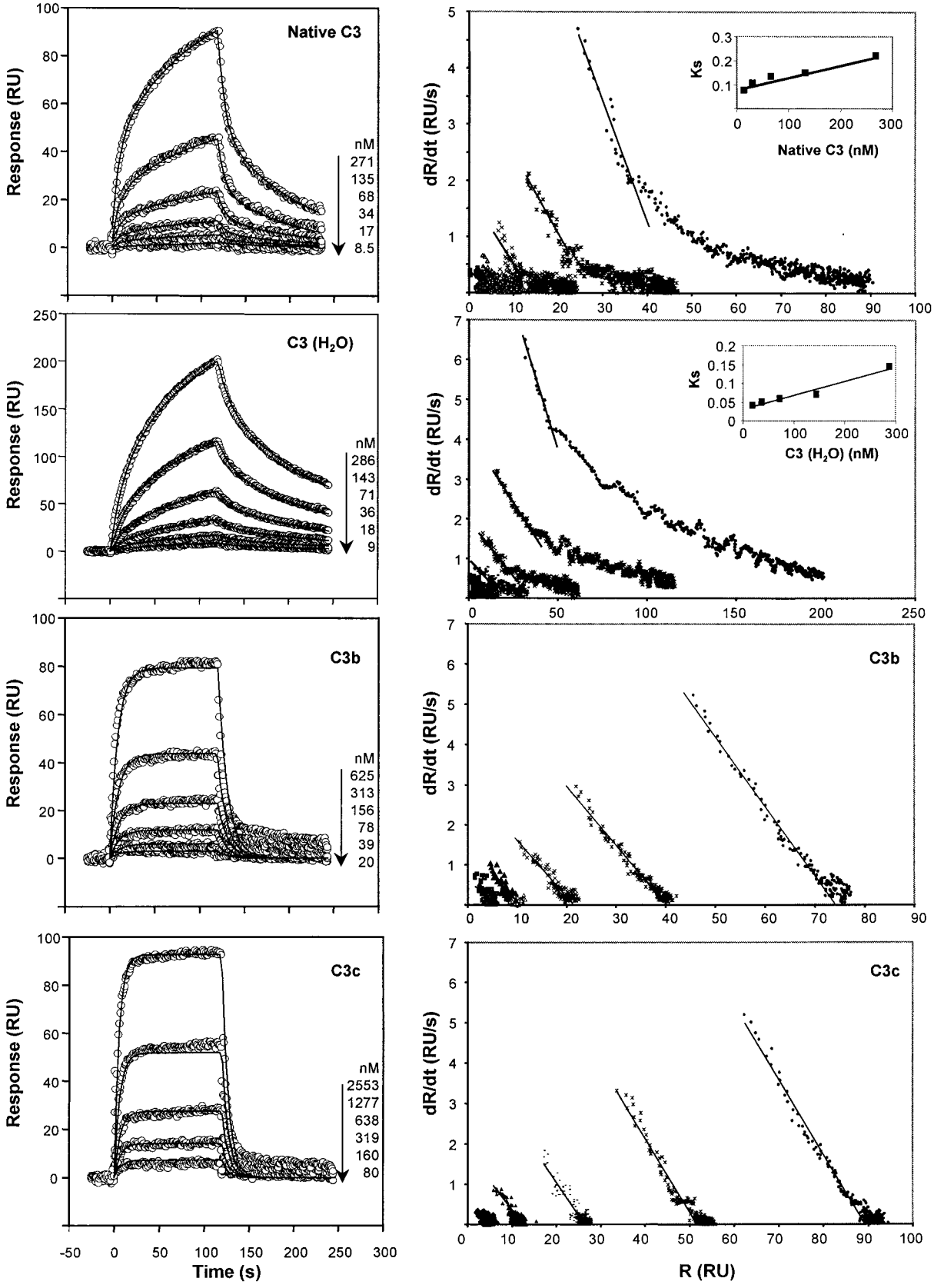

FIGURE 2. Analysis of the binding of Compstatin to C3 and C3 fragments by SPR. *Left*, Sensogram overlays for interactions between immobilized biotinylated Compstatin and native C3, C3(H_2O), C3b, and C3c. The concentration of analyte injected is indicated at the right of each sensogram overlay. Solid lines correspond to the global fitting of the data simultaneously. Native C3 and C3(H_2O) data were fit to a two-step conformational change model ($A + B \leftrightarrow AB \leftrightarrow AB^*$, BIAevaluation 3.0), whereas C3b and C3c data were fit to a 1:1 Langmuir binding model ($A + B \leftrightarrow AB$, BIAevaluation 3.0). *Right*, Linear transformations of the association phase data for the respective sensogram data shown on the left. The straight lines are linear least squares fits to the data. *Inset*, values of k_s (determined from the slope of the fits) replotted against analyte concentrations.

Table II. *Kinetic and affinity data for the Compstatin-C3 interaction*

Analyte	k_{on}[a] (10^5/s · M)	k_{off}[a] (10^{-2}/s)	K_D[b] (10^{-6} M)	Relative Affinity
Native C3	k_{on1} 5.0	6.6	K_{D1} 0.13	1
C3(H_2O)	k_{on1} 4.0	2.4	K_{D1} 0.06	0.46
C3b	0.34	9.4	2.8	21.5
C3c	0.15	14.2	9.6	73.8
C3d[c]	NA[d]	NA	NA	NA

[a] k_{on} and k_{off} for native C3 and C3(H_2O) were calculated by linear transformation of the respective data, whereas for C3b and C3c they were determined by global fitting of the data using a 1:1 Langmuir binding model.
[b] K_D was calculated from k_{off}/k_{on}.
[c] No binding was observed even at 7.1 μM.
[d] NA, Not applicable.

in human blood resulted in its rapid cleavage at the N terminus and generation of Compstatin (2–13) (Fig. 6). The second slow cleavage occurred between Arg^{11} and Cys^{12}. No cleavage was seen at the C terminus, because it was protected with amide. To determine whether the cyclic nature of the peptide blocks further processing, we incubated a linear analog (peptide III, Table I, in which Cys^2 and Cys^{12} were replaced with Ala) with human blood. It is clear from the data (Fig. 6, *B2*) that the linear analog was efficiently processed from the N-terminal end.

In an effort to increase the stability of Compstatin, we then blocked the α-amino group by acetylation. We reasoned that blocking of the amino terminus might protect the molecule from cleavage at Ile^1. As shown in Fig. 6, acetylation of the amino terminus blocked this cleavage. Further analysis of the acetylated Compstatin (Ac-Compstatin) showed that it was stable in human blood (half-life of 24 h at 37°C). These experiments were also repeated using normal human plasma and serum; the results were essentially similar to those obtained with human blood.

Functional activity of the biotransformation products of Compstatin

As described above, biotransformation of Compstatin in human blood resulted in its cleavage into a major product, Compstatin (2–13) and a minor linearized product, Compstatin (2–13) cleaved between Arg^{11} and Cys^{12}. Large quantities of these products were generated and purified by HPLC over a C-18 reversed-phase column (Vydac) and tested for their functional activity in a complement-mediated hemolytic assay. Compstatin (2–13) was 2-fold

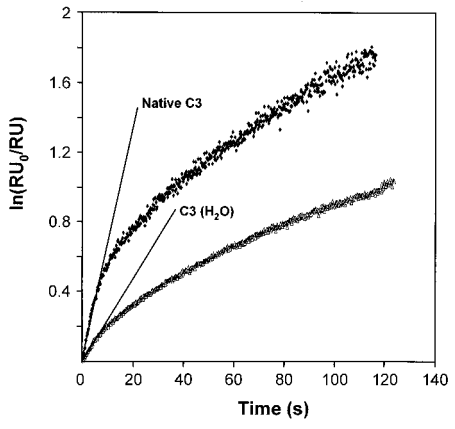

FIGURE 3. Kinetic analysis of the dissociation of Compstatin from native C3 and C3(H_2O). The dissociation phase data for the highest concentration of native C3 (271 nM) and C3(H_2O) (286 nM) were subjected to linear transformation. The slope of the straight line provided the off-rate.

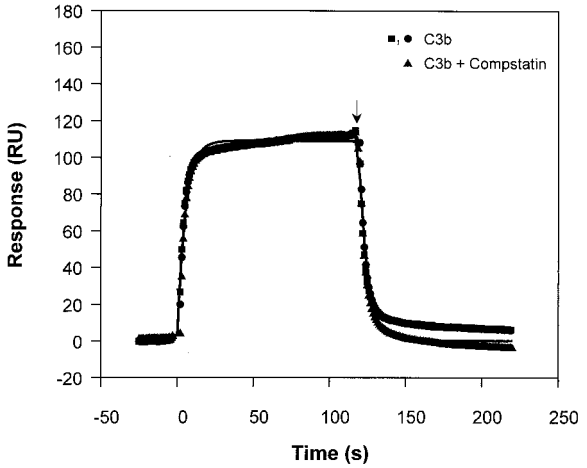

FIGURE 4. Coinjection of free Compstatin during the dissociation of C3b from immobilized Compstatin. A solution of C3b (625 nM) was flown over a streptavidin sensor chip containing a biotinylated analog (peptide XXIII) for 120 s after which it was replaced with the running buffer or running buffer containing 110 μM Compstatin (arrow) and followed for the next 100 s. The solid line is generated by fitting the data to a 1:1 Langmuir binding model.

less active than Compstatin, and its further cleavage between Arg^{11} and Cys^{12}, which linearized the peptide, resulted in the formation of an inactive species (Table I). These data emphasize that the cyclic nature of the peptide is important for its functional activity. Acetylation of the amino terminus blocked the cleavage of Ile^1; therefore, Ac-Compstatin was expected to have increased complement inhibitory activity in serum. Indeed, when it was tested in the hemolytic assay, Ac-Compstatin was three times more active than the unprotected Compstatin (Fig. 5 and Table I).

Discussion

The role of inappropriate complement activation in causing tissue damage in many clinical conditions (32) underscores the need for a specific complement inhibitor with therapeutic benefits. Complement inhibitors that are being developed include human complement control proteins (33–35), Abs to complement components

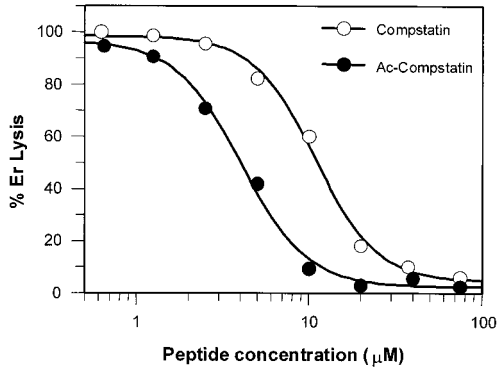

FIGURE 5. Inhibition of complement-mediated lysis of erythrocytes by various concentrations of Compstatin and Ac-Compstatin. Inhibition of complement activity by Compstatin and Ac-Compstatin was studied by measuring their effect on alternative pathway-mediated lysis of Er. Er were incubated for 20 min with NHS containing MgEGTA and various concentrations of peptides. The amount of lysis was determined by centrifuging the cells and measuring the absorbance of the supernatant at 405 nm. The data were normalized by considering 100% lysis to be equal to the lysis occurring in the absence of the peptide.

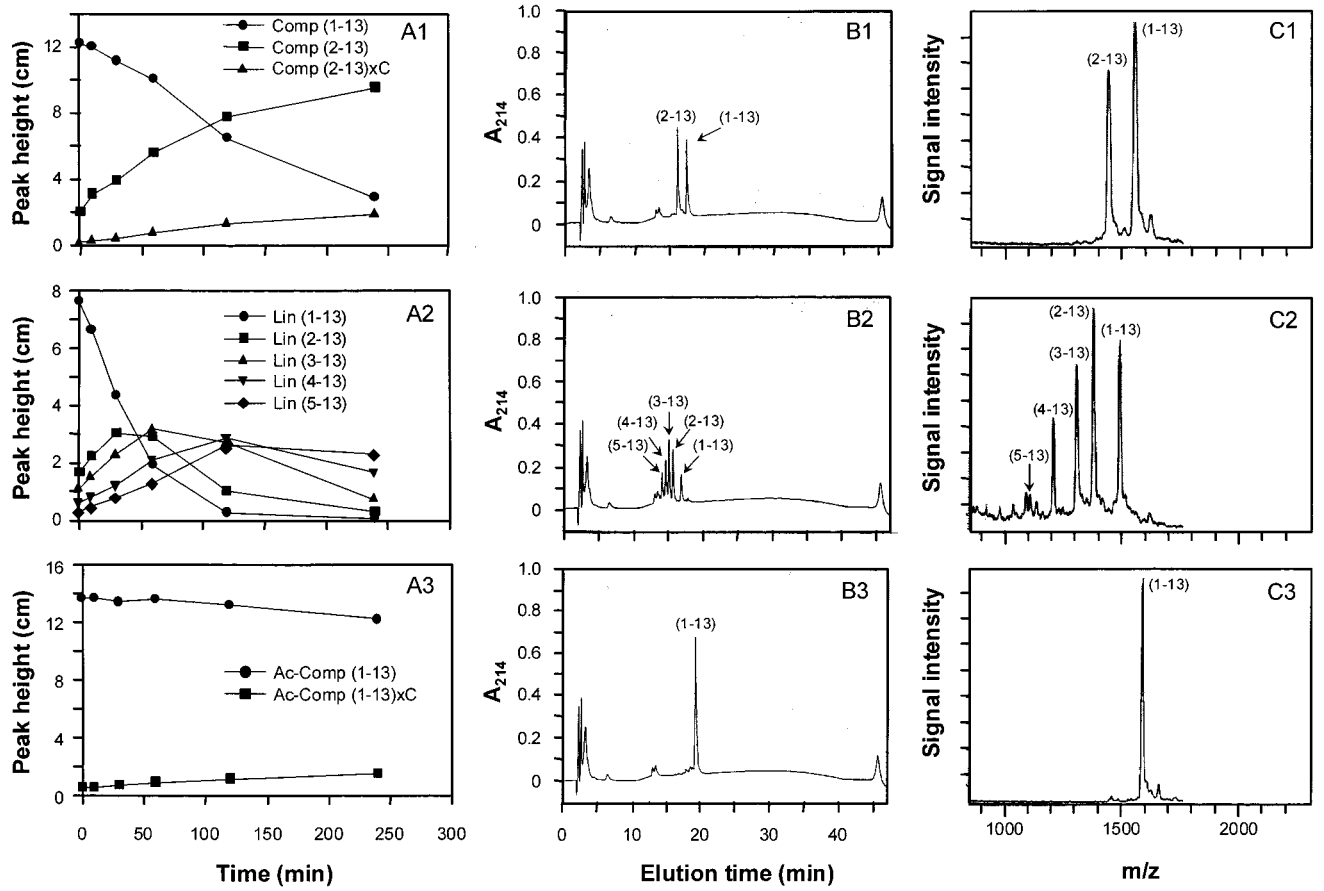

FIGURE 6. In vitro biotransformation of Compstatin and its analogs in human blood. *Left* (*A1–A3*), Time course of the biotransformation of Compstatin (Comp, peptide I), a linear analog (Lin, peptide III), and Ac-Compstatin (Ac-Comp, peptide V) in human blood. For each analog, the number in the parenthesis denotes the length of the peptide, and "xC" denotes the cleavage between Arg^{11}-Cys^{12}. Peak heights were determined from HPLC profiles. *Middle* (*B1–B3*), HPLC profiles identifying transformed products. *Right* (*C1–C3*), Matrix-assisted laser desorption spectra identifying transformed products.

(36), and protease inhibitors (37). Our efforts to target C3 led to the identification of a 13-aa cyclic peptide, Compstatin, that binds to C3 and inhibits its activation (14). In this study, we have further characterized the C3-Compstatin interaction. We have shown that 1) binding of Compstatin to native C3 is a multistep reaction that is preferred over its binding to the C3 fragments C3b and C3c; 2) the type I β-turn residues of Compstatin are not only important for conformational stability but are also involved in C3 binding; and 3) *N*-acetylation of Compstatin provides stability against enzymatic degradation.

Binding of Compstatin to C3

Analysis of the binding of Compstatin to C3 and its fragments C3b, C3c, and C3d (Fig. 2) indicated that the binding site is located in the C3c region. These results are in good agreement with previously published binding data obtained by ELISA (14). It is interesting that in our earlier study we observed that Compstatin displayed on the phage surface would bind to C3c when C3c was immobilized on a microtiter plate, but not when Compstatin was adsorbed to the plate and C3c was presented in solution (14). Based on this finding, we speculated that the binding site is not exposed in C3c unless the conformation of C3c is altered, e.g., by adsorption to a solid support (14). The SPR data presented here (Fig. 2, Table II) show that Compstatin binds to C3c, though with a 72-fold lower affinity than that of native C3. These data indicate that the binding site is clearly exposed in C3 as well as in its physiologically generated fragments C3b and C3c. We suggest that the lack of binding of C3c to immobilized Compstatin in the ELISA could have been due to its lower affinity for C3c, whereas the binding of phage-displayed peptide to immobilized C3c could be attributed to the presence of multiple copies of the peptide expressed on the phages (five copies per phage).

SPR analyses make it possible to probe the mechanism of protein-protein interaction in this system. Our real-time kinetic binding data from SPR measurements indicate that binding of Compstatin to C3 does not follow a simple 1:1 binding model (χ^2 = 15.5). Linear transformation of the data showed that the reaction is multistep (Fig. 2). Such deviations could simply result from heterogeneity in the surface and/or analyte being tested. However, we have ruled out both of these causes. First, homogeneity of the ligand surface was assured by orienting Compstatin in one direction. Further support came from the fact that the binding to C3b and C3c followed a 1:1 binding model (Fig. 2). Second, spontaneous hydrolysis of the thioester of C3 converts native C3 into C3(H_2O) (0.005% per min at 37°C) (28); thus, C3 preparations are mixtures of native C3 and C3(H_2O). For SPR analyses, native C3 was separated from C3(H_2O) before the experiment and kept at 0°C until assay, and the protein sample was reanalyzed at the end of the experiment to detect the presence of C3(H_2O); <2% of the C3 was hydrolyzed to C3(H_2O). Therefore, the nonlinear *dR*/*dt* plot shown in Fig. 2 could mean genuine multistep binding. Such plots could reflect the presence of multiple binding sites with different affinities, a conformational change, or a more complex model. Experiments designed to localize the Compstatin binding

site on C3 indicated that the binding site is located within the 40-kDa C-terminal half of the β-chain of C3 (38). Thus, it is unlikely that the nonlinear *dR*/*dt* plot we obtained is related to the presence of multiple binding sites. Moreover, the data did not fit well to a bivalent analyte model ($A + B \leftrightarrow AB$, $AB + B \leftrightarrow AB_2$, $\chi^2 = 13$). The possibility that Compstatin undergoes a structural reorientation upon binding remains open. The occurrence of conformational changes in peptides upon binding has been reported for cyclosporine complexed to cyclophilin (39, 40) and for the Bak peptide complexed to the antiapoptotic protein Bcl-x_L (41). To determine whether our data supported this possibility, we tried to fit our data to a two-state conformational change model ($A + B \leftrightarrow AB \leftrightarrow AB^*$). The data fit well with this model (see fit, Fig. 2; $\chi^2 = 1.57$). Currently, studies of the bound peptide are underway to verify this premise.

Cleavage of native C3 by C3 convertases results in generation of C3b (185 kDa) and C3a (9 kDa). C3b possesses the ability to bind to a large number of serum and membrane proteins (42). The binding sites for these proteins are buried in native C3 and become available only after a conformational change that occurs upon cleavage of C3 to C3b. Like C3b, C3(H_2O) binds to these serum and membrane proteins. In addition, both C3b and C3(H_2O) show enhanced binding to the fluorophore 8-anilino-1-nephthalensulfonate and a similar positive shift in near-UV circular dichorism spectrum when compared to C3 (27). Based on these results, it is largely considered that C3b and C3(H_2O) have similar conformations. The data presented in Fig. 2 shows that binding of Compstatin to C3(H_2O) is biphasic, whereas that to C3b follows a 1:1 binding model. Thus, our results indicate that at least the conformation of the Compstatin binding site in C3(H_2O) is different from that of C3b.

Structure-activity relationships in Compstatin

Earlier analysis of the functional activity of Compstatin showed that reduction and alkylation of Compstatin destroys its inhibitory activity in serum, a finding that indicated that oxidation of cysteine residues is required for its functional activity (14). However, the reason for this loss of activity was not clear. By using a direct binding SPR assay we have now shown that linearization of Compstatin results in the loss of its ability to bind to C3, a result that suggests that oxidation of cysteine maintains the structure of Compstatin that is optimal for binding and thus preserves its functional activity.

The interaction between a peptide and its receptor/binding protein can involve an interaction between side-chain and main-chain atoms. Retro-inverso peptidomimetic analogs are attractive tools for probing the relative contributions of both sets of atoms to a bimolecular recognition process. In such analogs, activity is preserved if the orientation of the side chain is the most important factor in the specific ligand interaction. For example, recent studies have demonstrated the preservation of the antigenicity of a retro-inverso peptide mimetic derivative of the C terminus of the histone H3 sequence IRGERA (43) and the transport function of a 16-mer retro-inverso form of a homeobox domain (44). In contrast, if main-chain atoms play a significant role in the ligand interaction, the analogs are inactive, as has been inferred from the analysis of a hormone-binding domain of a vasopressin receptor (45). In our current study, the retro-inverso mimetic of Compstatin was inactive (Table I); thus, it appears that binding to C3 is not solely dependent on the side chain interactions and that correctly oriented main-chain atoms also contribute to binding.

Turn structures, which have been recognized for >30 years (46), are intrinsically polar in nature, with the backbone groups packed together and the side chains projected outward. Turns are often considered potential sites for molecular recognition. In small peptides, they are known to provide conformational stability by optimizing backbone-chain compactness and to facilitate intermolecular recognition through side-chain clustering (31). Compstatin contains a type I β-turn encompassing residues Gln^5-Gly^8. Alanine scan studies of Compstatin analogs have suggested that these residues are important for the inhibitory activity of the peptide (15). However, from these results it was not clear whether the β-turn is critical only for preservation of the structural stability or whether it is also important for intermolecular recognition. We have now addressed this question by synthesizing β-turn substitution analogs. Peptide analogs in which the β-turn segment was replaced with residues having the tendency to form a type I turn (Gly^5-Pro^6-Phe^7-Gly^8, peptide XVII) (31) or type II turn (Gly^5-Pro^6-DPhe^7-Gly^8, peptide XXI) (31), and analogs in which individual turn residues were substituted (Gln^5-Gly and Trp^7-Phe) were functionally inactive (Table I). Therefore, it appears that side-chain interactions exist between some of the turn residues and C3.

Biotransformation of Compstatin

The proteolytic susceptibility of a biologically active peptide is as important to its biological effect as its affinity for the target protein and its in vivo half-life. To determine the susceptibility of Compstatin to proteolytic cleavage by serum proteases, we incubated this peptide in human blood. Under these conditions, Compstatin was processed rapidly at the N terminus, resulting in the removal of Ile^1. However, the N-terminal processing apparently stopped after the first amino acid residue. One explanation, although unlikely, is that cleaved products were produced but were not detected because of selective loss during the sample preparation. However, under the same condition, the linear analog of Compstatin was successively cleaved at the N terminus, and all of the transformed products were detected (Fig. 6). Therefore, it is very unlikely that Compstatin products were not detected because of a selective loss on the filter membranes. Thus, our data demonstrate that the cyclic nature of Compstatin is important not only in stabilizing its structure but also in protecting it from enzymatic processing. To our knowledge, Compstatin is the first example of a peptide whose proteolytic processing in blood has been shown to be blocked by cyclization.

In the past, many attempts have been made to develop synthetic peptides as complement inhibitors. These peptides were based on the C3 convertase cleavage site in C3 (47), the factor D cleavage site in factor B (48), sites of length polymorphism in C3 or C5 (49), or the C terminus of the serine protease inhibitor (48). None of these approaches has yielded a potent peptide that can inhibit complement activation. One possibility that must be considered to explain the failure of these approaches is the biotransformation of the peptides in serum. Fig. 6 demonstrates that linear peptides are very susceptible to protease processing. In the above-mentioned studies, the inhibitory activities of the peptides were tested in complement-mediated hemolytic assays that use serum as a source of complement. Therefore, it is possible that failure of these approaches could be explained at least in part by the processing of the peptides by serum proteases.

In this study we have shown that acetylation can prevent the N-terminal processing of Compstatin (removal of Ile^1) and thereby enhance the inhibitory activity of the peptide. The acetylated analog had an inactivation rate of 0.01%/min at 22°C (Fig. 6) and 0.03%/min at 37°C (data not shown) in serum. These data indicate that Ac-Compstatin is stable against proteolytic cleavage in human blood and has the potential to be developed as an in vivo complement inhibitor.

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