Hydrophobic Effect and Hydrogen Bonds Account for the Improved Activity of a Complement Inhibitor, Compstatin

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Tryptophans at positions 4 and 7 of compstatin, a peptide complement inhibitor, are crucial for its interaction with C3. However, the nature of their involvement has not been studied to date. Here we investigate the molecular forces involved in the C3−compstatin interactions, mediated by aromatic residues, by incorporating in these two positions various tryptophan analogues (5-methyltryptophan, 5-fluorotryptophan, 1-methyltryptophan, and 2-naphthylalanine) and assessing the resulting peptides for activity by enzyme-linked immunosorbent assay (ELISA) and binding by isothermal titration calorimetry (ITC). Of all the compstatin analogues, peptides containing 1-methyltryptophan at position 4 exhibited the highest binding affinity (K_d = 15 nM) and activity (IC_{50} = 0.205 μM), followed by a peptide containing 5-fluorotryptophan at position 7. Our observations suggest that hydrophobic interactions involving residues at position 4 and the hydrogen bond initiated by the indole nitrogen are primarily responsible and crucial for the increase in activity. These findings have important implications for the design of clinically useful complement inhibitors.

Introduction

Compstatin is a 13-amino acid cyclic peptide inhibitor that binds to the β-chain of C3,[1] a complement component that is central to all three pathways of the complement system (classical, alternative, and lectin).[2] A number of inhibitors ranging from small organic compounds to large-size proteins have been designed to act at several points in the complement cascade, but compstatin, a 13-amino acid cyclic peptide (ICV-VQDWGHHRCT-NH_2), is unique in acting at the level of C3.[3] Compstatin has been tested successfully in several in vivo and ex vivo animal models and has shown enormous potential for development as a complement therapeutic.[4−7] The three-dimensional structure of the peptide, as determined by NMR, shows a coil with a type II β-turn involving Q5-D6-W7-G8.[8]

With this structure as a template, several combinatorial, computational, and rational design approaches have been carried out to enhance the activity of compstatin.[10,11] These enhancements have yielded a peptide, having 2-naphthylalanine in position 4, with 99-fold higher activity than that of the parent molecule. Several of the most active analogues have contained tryptophan or other aromatic ring structures at positions 4 or-(and) 7, underscoring the importance of an aromatic ring structure at these positions for complement inhibitor activity.[12]

Despite this increase in activity, very little is known about the underlying molecular forces that impart recognition and specificity to compstatin and thereby dictate its inhibitory activity. Incorporation of aromatic residues such as tyrosine at position 4 results in a lower activity than that seen when tryptophan is present.[10,11] The fact that tryptophan and tyrosine differ in the electrostatic potentials of their ring suggested a possible correlation between the ring current and the inhibitory activity; indeed, an increase in inhibitory activity was observed with an increase in the negative electrostatic potential. Such a relationship is indicative of a cation−π interaction, as has been described for several protein−protein interactions.[13−18] In addition, the higher hydrophobicity of tryptophan when compared to tyrosine may also be responsible for the higher activity observed.

Compstatin contains a tryptophan residue at position 7 that participates in the formation of the β turn.[8] Substitution of this tryptophan residue with alanine or phenylalanine results in loss of activity,[9] thus indicating that tryptophan is crucial at this position for the structural stability and/or activity of compstatin. Loss of activity upon substitution by phenylalanine indicates that Trp7, in addition to Trp4, may mediate a cation−π interaction or the formation of a hydrogen bond between the N−H and a hydrogen-bond acceptor on C3. However, the role of Trp7 in these interactions has not been delineated at the atomic level.

In this study we have used peptide synthesis and isothermal titration calorimetry (ITC)[a] analysis to examine the potential noncovalent interactions involved in this interaction between C3 and compstatin and to analyze the role of selective hydrogen bonding at positions 4 and 7. Analyses in which we introduced the tryptophan analogues 5-fluorotryptophan, 5-methyltryptophan, 1-methyltryptophan, and 2-naphthylalanine (Figure 1) at positions 4 and/or 7 have revealed that the incorporation of 1-methyltryptophan at position 4 yields a peptide with a 264-fold increase in inhibitory activity. Our results suggest that reducing the hydrophobic character of Trp4 results in a dramatic loss in inhibitory activity. In contrast, at position 7, hydrophobic residues are not tolerated, and they drastically lower the inhibitory activity. Instead, an interaction of a polar nature, such as hydrogen bonding, is preferred, and increasing the number of hydrogen bonds increases the inhibitory activity of compstatin analogues.

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[a] Abbreviations: Ac, acetyl group; ELISA, enzyme-linked immunosorbent assay; 5fW, 5-fluoro-L-tryptophan, 1MeW, 1-methyl-L-tryptophan; 5MeW, 5-methyl-L-tryptophan; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; HPLC, high-performance liquid chromatography; ITC, isothermal titration calorimetry; 2-Nal, 2-naphthylalanine; NA, not active; log *P*, logarithm of partition coefficient.

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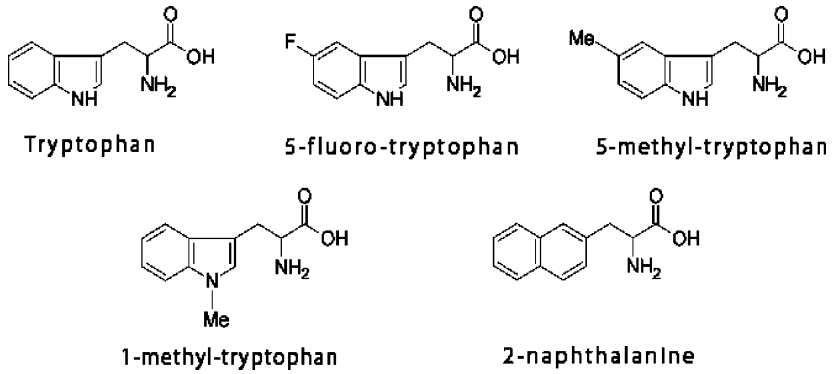

Figure 1. Side-chain structures of analogues used in the study: (A) tryptophan (W), (B) 5-fluorotryptophan (5fW), (C) 5-methyltryptophan (5MeW), (D) 1-methyltryptophan (1MeW), and (E) 2-naphthylalanine (2Nal).

Results

Synthesis of Compstatin Analogues. The analogues of compstatin were synthesized by solid-phase Fmoc-based peptide chemistry. Because of the unavailability of Fmoc-derived L-derivatives of the tryptophan analogues, the Fmoc-derived DL-derivatives were used. The reactions yielded a diastereomeric mixture of peptides bearing a D-analogue or an L-analogue. Therefore, each of the peptide mixtures was further subjected to reversed-phase HPLC to separate the peptide diastereomers, as described under Materials and Methods. Each peptide with a single substitution yielded two well-separated peaks, while the peptide with substitutions at both positions 4 and 7 yielded four well-separated peaks. Digesting the peptides corresponding to these peaks with V8 protease and subsequent MALDI analysis made it possible to identify the diastereomeric nature of the analogues containing tryptophan analogues at position 7. The rationale for using V8 protease was that this enzyme cleaves at the C-terminal side of aspartic acid only when followed by an L-amino acid. Identification of cleavage products in the mass spectra indicated that the L-diastereomeric peptide eluted first, followed by the D-form(s), for which no cleavage fragments were detected (data not shown). On the basis of these observations, we concluded that the peptide eluted in the first peak is of the L-form and in the second peak is of the D-form. This conclusion was further supported by assessment of the complement-inhibiting activity of the peptides eluted in the two peaks. In agreement with previous findings, where any D-amino acid substitution renders the molecule inactive,[9] the second peak showed no activity while the first peak inhibited complement activation (data not shown).

Incorporation of Tryptophan Analogues at Position 4. It has previously been shown that substitution of valine with tryptophan at position 4 increases the activity of compstatin by 45-fold.[11] In the present study we replaced tryptophan with tryptophan analogues and 2-naphthylalanine to study the nature of the interaction mediated by the residue at position 4 during the course of the binding of compstatin analogues to C3. Previously described ELISA-based assays were used to test the activity of all the compstatin analogues bearing tryptophan analogues at position 4. While substitution with 1-methyltryptophan (1MeW), 5-methyltryptophan (5MeW), and 2-naphthylalanine (2Nal) increased the activity of compstatin 264-, 67-, and 99-fold, respectively, substitution with 5-fluorotryptophan (5fW) resulted in a lower activity: 31-fold increase (Figure 2, Table 1). Figure 4A shows inhibitory constants (IC_{50}) plotted against log *P* values for the tryptophan analogues and 2-naphthylalanine. The plot indicates that the activity of compstatin increases with an increase in the hydrophobicity of the amino acid replaced at position 4. However, the IC_{50} representing the activity of compstatin analogue with 1-methyltryptophan [4(1MeW)7W] did not fall on the linear line, showing an exception to this correlation.

To investigate whether a correlation exists between the activity and binding affinity, the interaction of the compstatin analogues listed in Table 1 was measured by isothermal titration calorimetry. The calorimetric data obtained for the interaction of all the peptides with C3 fit to "one set of sites" model, with a stoichiometry close to 1 (Figure 3). These results suggest that the binding of these peptides to C3 occurs in a 1:1 ratio. The thermodynamic parameters resulting from these fits are shown in Table 2. As evident from the K_d values, peptide 4(1MeW)-7W exhibited higher binding affinity ($K_d = 0.015$ μM) than any of the other peptides listed in category I. Plotting these values against the log *P* values of analogues indicated that a correlation exists between the binding affinity and the hydrophobic nature of the tryptophan analogues (except the 1-methyltryptophan analogue) and 2-naphthylalanine (Figure 4B): the binding affinity increased with an increase in the hydrophobicity of the analogue incorporated at position 4. This observation is consistent with the correlation observed between log *P* and the inhibitory constants, indicating that the activity follows binding affinity. For example, increase in the activity observed for 4(1MeW)7W in comparison to 4W7W is mainly due to its increased binding affinity to C3. Consistent with the correlation observed between log *P* and IC_{50}, the exception exhibited by 4(1MeW)7W is reiterated in the log $P-K_d$ correlation. This indicates that the anomalous behavior of 4(1MeW)7W is not due to the nature of inhibition assays or binding constant determinations.

All the peptides bound to C3 with a negative enthalpy and positive entropy, suggesting that the binding is enthalpy-driven. Such binding was previously shown to be characteristic for the interaction of compstatin with C3.[19] However, binding of all the compstatin analogues in category I was characterized by an enthalpy change that was lower than that observed for 4W7W and by an entropy change shifted toward the favorable end. Figure 4C shows a plot of log *P* versus $-T\Delta S$, which indicates that, with an increase in the hydrophobicity of the analogues incorporated at position 4, entropy is more favored; this situation has a positive impact on the free energy change (Table 2), which is consistent with an increase in binding affinity.

Incorporation of Tryptophan Analogues at Position 7. It has been proposed in previous studies that tryptophan at position 7 may form a hydrogen bond with a H-bond acceptor on C3. In the present study we therefore replaced tryptophan at position 7 (4W7W) with tryptophan analogues such as those used for

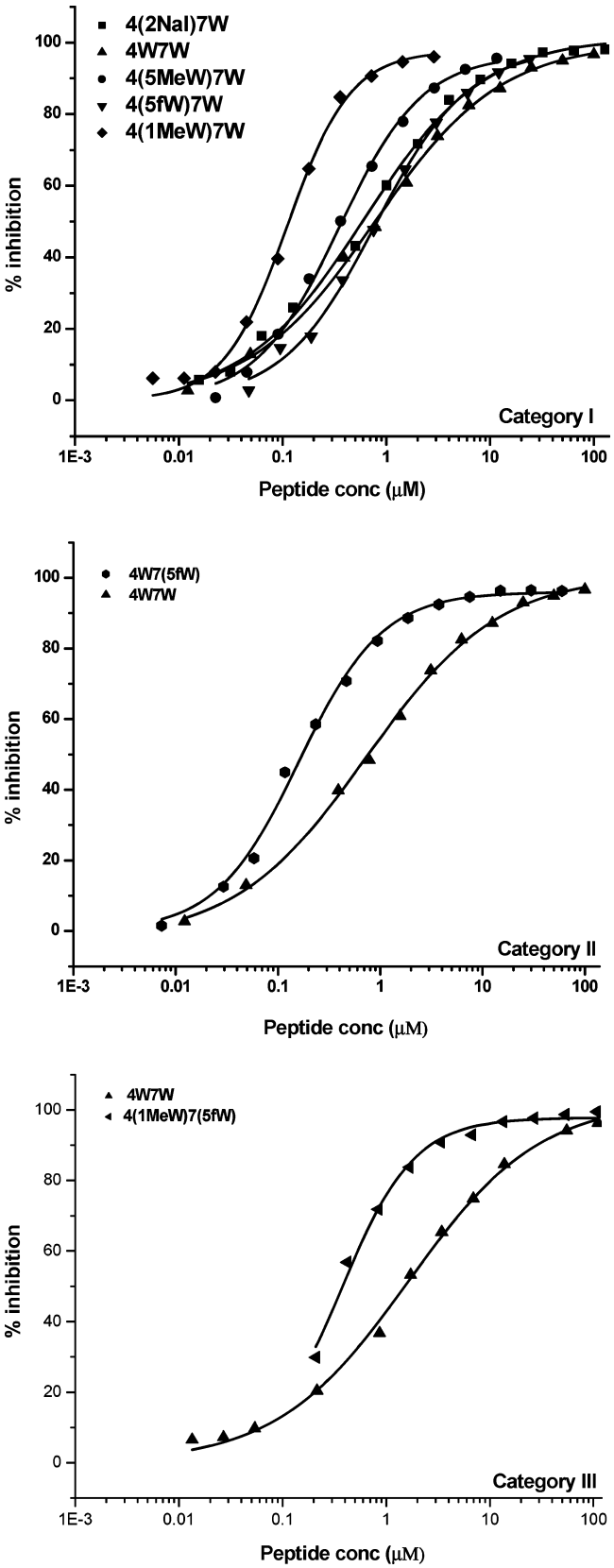

Figure 2. Activity of synthetic compstatin analogues: Plots of percent complement inhibition vs peptide concentration for 4W7W compstatin (▲), 4(5fW)7W (▼), 4(5MeW)7W (●), 4(1MeW)7W (◆), 4(2Nal)7W (■), 4W7(5fW) (⬢), and 4(1MeW)7(5fW) (triangles pointing left).

position 4. With the exception of the substitution with 5-fluorotryptophan, which yielded a 121-fold active peptide, all the other substitutions rendered compstatin inactive (Figure 2, Table 1).

Table 1. Sequences of Peptides Used in This Study

peptide	sequence	IC_{50} (μM)	RA[a]
4W7W	Ac-ICVWQDWGAHRCT-NH_2	1.2	45
	Substitution at Position 4 (Category I)		
4(5fW)7W	Ac-ICV(5fW)QDWGAHRCT-NH_2	1.74	31
4(5MeW)7W	Ac-ICV(5MeW)QDWGAHRCT-NH_2	0.87	67
4(1MeW)7W	Ac-ICV(1MeW)QDWGAHRCT-NH_2	0.205	264
4(2Nal)7W	Ac-ICV(2Nal)QDWGAHRCT-NH_2	0.545	99
	Substitution at Position 7 (Category II)		
4W7(5fW)	Ac-ICVWQD(5fW)GAHRCT-NH_2	0.446	121
4W7(5MeW)	Ac-ICVWQD(5MeW)GAHRCT-NH_2	NA	NA
4W7(1MeW)	Ac-ICVWQD(1MeW)GAHRCT-NH_2	NA	NA
	Double Substitution at Positions 4 and 7 (Category III)		
4(1MeW)7(5fW)	Ac-ICV(1MeW)QD(5fW)GAHRCT-NH_2	0.205	264

[a] Activity relative to the peptide H-I(CVVQDWGHHRC)T-NH_2.

Thus, unlike the activity exhibited by the peptides listed in category I, no correlation was observed between the activity and hydrophobicity of the tryptophan analogues at position 7.

Calorimetric titration of the peptides listed in category II yielded the thermodynamic parameters listed in Table 2; since no binding was detected for peptides bearing 5-methyltryptophan and 1-methyltryptophan, binding parameters do not exist for these analogues. Only 4W7(5fW) bound to C3, with an affinity of 0.035 μM; this value is greater than that observed for any of the peptides in category I except 4(1MeW)7W. In contrast to the peptides in category I, 4W7(5fW) bound to C3 with a favorable binding enthalpy change ($\Delta H = -21.83$ kcal/mol, $\Delta\Delta H = -3.69$ kcal/mol) and unfavorable entropy change ($-T\Delta S = 11.56$ kcal/mol, $-T\Delta\Delta S = 2.77$ kcal/mol), suggesting the involvement of additional favorable noncovalent interactions of polar nature.

Incorporation of Tryptophan Analogues at Both Positions 4 and 7. Since the substitution of tryptophan with 1-methyltryptophan at position 4 and with 5-fluorotryptophan at position 7 yielded compstatin analogues that showed a marked increase in inhibitory activity, we also created an analogue bearing simultaneously both these substitutions. The resulting peptide, 4(1MeW)7(5fW), generated an inhibition curve similar to that for the single substitution with 1-methyltryptophan [4(1MeW)-7W] (Figure 2, Table 1). The binding affinity ($K_d = 0.017$) observed for the double analogue was also similar to that for 4(1MeW)7W. Binding of 4(1MeW)7(5fW) to C3 yielded an enthalpy change of -17.33 kcal/mol, an entropy change of -6.73 kcal/mol, and a free energy change of -10.6 kcal/mol. These values are similar to those observed for the binding of 4(1MeW)7W suggesting that 5-fluorotryptophan has no effect at position 7 in the presence of 1-methyltryptophan at position 4.

Discussion

Hydrogen Bonds at Position 7 Increase the Activity of Compstatin. Tryptophan at position 7 is one of the four residues that make up the β turn, which has been shown to be critical for the activity of compstatin.[9] It has also been suggested that Trp7 might stabilize the interaction of compstatin with C3 through a hydrogen bond. However, no direct experimental evidence has been obtained to support this hypothesis. In the present study, incorporation of 5-fluorotryptophan at this position resulted in an increase in the inhibitory activity, whereas incorporation of either 5-methyltryptophan or 1-methyltryptophan rendered compstatin inactive. This loss of activity upon incorporation of 1-methyltryptophan supports the contention that

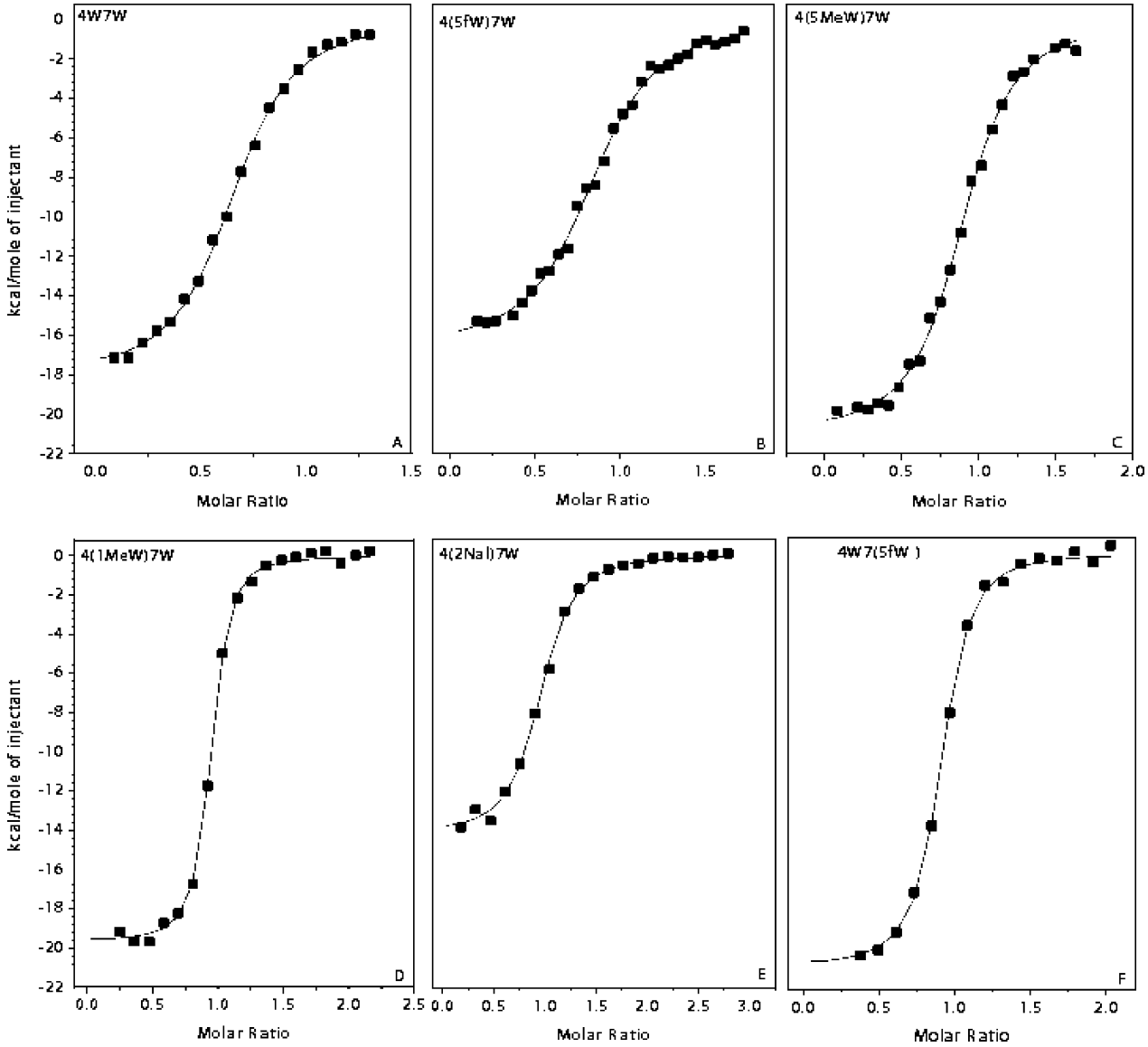

Figure 3. Thermodynamic characterization of the interaction of compstatin analogues with C3: ITC data representing the binding of 4W7W, 4(5fW)7W, 4(5MeW)7W, 4(1MeW)7W, 4(2Nal)7W and 4W7(5fW) to C3. The plots were obtained by fitting the corrected raw data to "one set of sites" model in Origin 7.0. All the errors reported in Table 2 are results of nonlinear least-squares fit.

Table 2. Thermodynamic Parameters for the Interaction of Synthetic Compstatin Analogues

peptide	K_a (M^{-1})	K_d (μM)	ΔH (kcal/mol)	$\Delta\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	$-T\Delta\Delta S$ (kcal/mol)	ΔG (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
4W7W	$7.036 \times 10^6 \pm 4.8 \times 10^5$	0.14	-18.14 ± 0.19	0	8.79	0	−9.4	0
4(5fW)7W	$6.65 \times 10^6 \pm 5.1 \times 10^5$	0.15	-16.69 ± 0.23	1.45	7.39	−1.4	−9.4	0
4(5MeW)7W	$9.59 \times 10^6 \pm 7.3 \times 10^5$	0.12	-17.75 ± 0.22	0.34	8.2	−0.54	−9.55	−0.15
4(1MeW)7W	$6.56 \times 10^7 \pm 6.6 \times 10^6$	0.015	-17.59 ± 0.14	0.81	6.94	−1.85	−10.65	−1.1
4(2Nal)7W	$9.21 \times 10^6 \pm 9.7 \times 10^5$	0.11	-14.27 ± 0.19	3.87	4.8	−3.99	−9.5	−0.1
4W7(5fW)	$2.83 \times 10^7 \pm 3.7 \times 10^6$	0.035	-21.83 ± 0.10	−3.69	11.56	2.77	−10.25	−0.8
4(1MeW)7(5fW)	$5.91 \times 10^7 \pm 4.7 \times 10^6$	0.017	-17.33 ± 0.093	0.81	6.73	−2.06	−10.6	−1.2

the hydrogen bond mediated by N−H of Trp 7 is crucial for the interaction of compstatin with C3. Since we were unable to observe any binding of the peptide containing 1-methyltryptophan with C3 in the calorimeter, the enthalpy change associated with the hydrogen bond formation could not be determined. In addition, the complete loss of activity of compstatin upon incorporation of 5-methyltryptophan strongly suggests that a hydrophobic amino acid is not tolerated at position 7, probably because of the polar nature of the interaction mediated at position 7.

Incorporation of 5-fluorotryptophan at position 7 increased the enthalpy by −3.69 kcal/mol when compared to 4W7W (Table 2). This finding suggests that enthalpically favored interactions are mediated by the fluorine atom. Replacing one of the indole hydrogens with a fluorine atom might strengthen the hydrogen-bonding character of the indole NH as a result of the drop in pK_a. However, the magnitude of the enthalpy increase was rather high to be attributable to an increase in the hydrogen-bonding strength of the indole NH. Another possibility is that the fluorine forms a hydrogen bond as a result of its

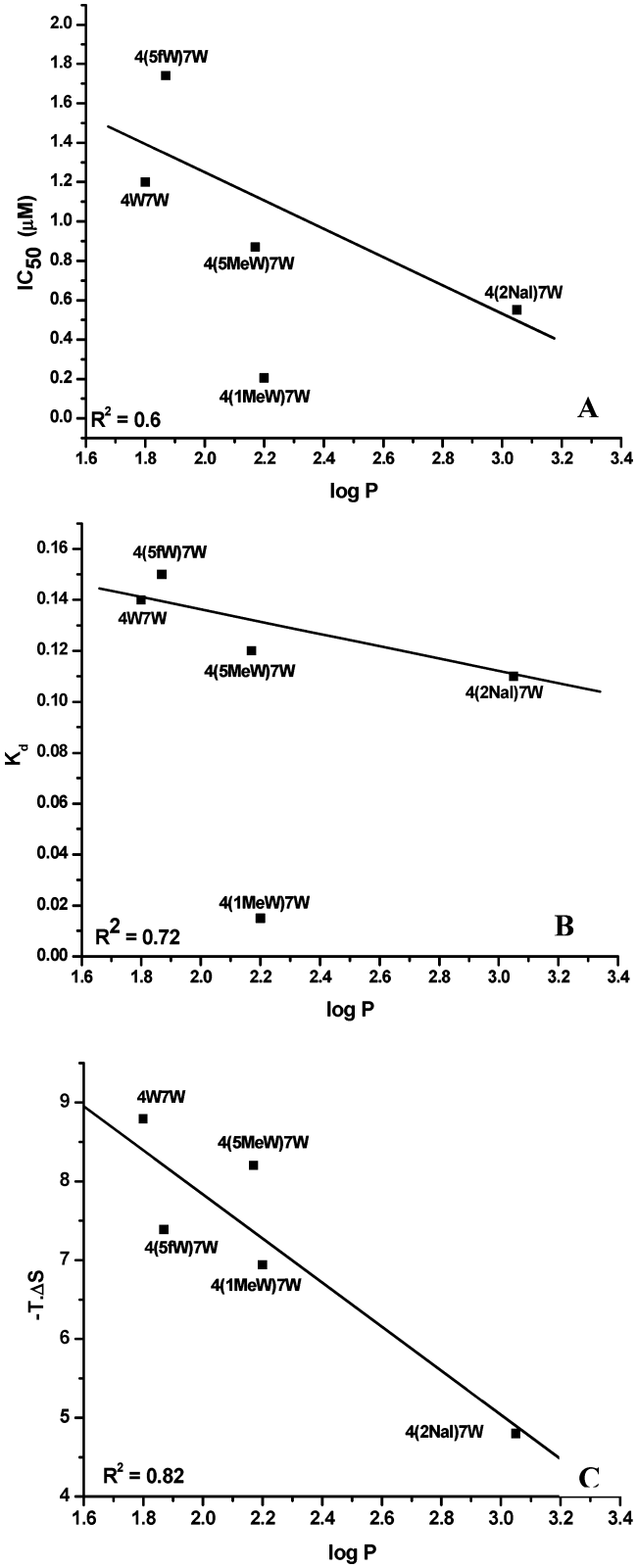

Figure 4. Plots showing the relationship between hydrophobicity of the analogues at position 4, denoted by log *P*, and the inhibitory constant (A), the binding constant (B), and entropy, denoted by $-T\Delta S$ (C).

electron-donating nature, as has been demonstrated for the structure of the tetradeca(3-fluorotyrosyl)glutathione transferase.[20] This possibility would more likely fit our observations, as the energy of the hydrogen bond is about 2 kcal/mol. However, the magnitude is twice the hydrogen-bond energy. One possible explanation is that a water molecule is bridging the interaction between the fluorine atom and a hydrogen acceptor on C3, in which case two hydrogen bonds (equivalent to about 4 kcal/mol energy) need to be formed. Our studies on the thermodynamics of the interaction of C3 with compstatin suggest that water molecules are involved at the binding interface.[19] Thus, the hypothesis that water mediates the interaction between the fluorine atom and a residue on C3 fits our observations. Further support comes from the decrease in entropy observed for the interaction of the position 7-substituted 4W7(5fW) analogue relative to the 4W7W analogue (Table 2), a decrease that could be produced by the binding of an additional water molecule at the interface. Such water-mediated interactions between fluorine atoms and other hydrogen bond acceptors have been observed in other systems.[21]

A Hydrophobic Effect at Position 4 Increases the Activity of Compstatin. It has been shown in computational studies and ELISA-based activity assays that substitution of valine with tyrosine at position 4 increased the activity of compstatin 14-fold.[10] Further substitution of valine with tryptophan increases the activity of compstatin 45-fold.[11] The basis for the difference in activity observed between the tyrosine- and tryptophan-containing peptides has not been studied. This difference, however, suggests that incorporation of an aromatic residue at position 4 makes compstatin more active. There are four possibilities to account for an increase in the activity exhibited by a tryptophan-containing peptide when compared to a tyrosine-containing peptide: (1) A hydrophobic effect at position 4 is required for the interaction, as tryptophan is more hydrophobic than tyrosine. (2) A cation$-\pi$ interaction could be initiated at position 4, as tryptophan possesses a greater π electron density than tyrosine. (3) A hydrogen bond could be formed between tyrosine or tryptophan and a residue on C3, and together with the above-mentioned interactions, this reaction could better stabilize the interaction. (4) Incorporation of analogues changes the structure of compstatin to the extent that the interaction is effected.

To investigate the possible involvement of these interactions or changes at position 4, we incorporated 5-methyltryptophan, 5-fluorotryptophan, 1-methyltryptophan, and 2-naphthylalanine at this position. Incorporation of 1-methyltryptophan produced the greatest increase in activity and binding affinity. This finding is in marked contrast to the result we obtained when we incorporated 1-methyltryptophan at position 7. This observation strongly suggests that an indole N-mediated hydrogen bond is not necessary at position 4 for the binding and activity of compstatin. In fact, the absence of this hydrogen bond, or a reduction of the polar character by replacing hydrogen with methyl on the indole nitrogen at position 4, seems to be beneficial for the binding and activity of compstatin. These findings provide strong evidence that a hydrophobic interaction or effect at position 4 strengthens the interaction of compstatin with C3. Further support for this conclusion comes from the log $P-\mathrm{IC}_{50}$ and log $P-K_\mathrm{d}$ correlations observed upon incorporation of 5-methyltryptophan, 5-fluorotryptophan, and 2-naphthylalanine, as well as the entropy differences observed upon incorporation of the analogues. In the classical hydrophobic effect, an increase in entropy is expected; in our case, such a result is clearly indicated by the plot showing the relationship between the hydrophobic nature of the incorporated analogues and the entropy (Figure 4). We suggest that this increase in entropy could be an effect of the bulk solvent.

Tryptophan has been shown to participate in cation$-\pi$ and $\pi-\pi$ interactions through the π electron density on the indole ring (Figure 5; tryptophan), and these interactions are believed

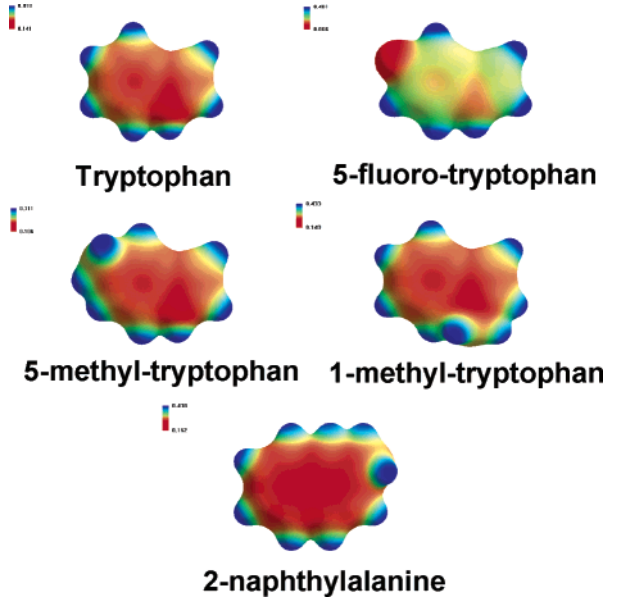

Figure 5. Representation of the electrostatic potential of the side chains of tryptophan, 5-fluorotryptophan, 5-methyltryptophan, 1-methyltryptophan, and 2-naphthylalanine. Red indicates negative potential, and blue indicates positive potential. These maps were created with Chemsketch.

to impart specificity to several intermolecular and intramolecular interactions involving proteins[13,14,18,22] and peptides[23,24] and also to catalysis.[16,17,22,25] Thus, reducing the π character of the indole by introducing strong electronegative groups such as fluorine (Figure 5; 5-fluoro-tryptophan) onto the indole ring, as in our case, could lead to a weakening of the cation$-\pi$ and $\pi-\pi$ interactions. Such changes in the strength of a cation$-\pi$ interaction as a function of the degree of fluorination of the indole ring have previously been demonstrated in the interactions between the acetylcholine receptor and nicotine and between the serotonergic receptor and serotonin.[15] In contrast, placement of electron-rich substituents such as methyl groups on the indole should leave the π electron density unperturbed (Figure 5; 5-methyl-tryptophan and 1-methyl-tryptophan) and would be expected to leave the π electron-mediated interactions unchanged. Similarly, the addition of a benzene ring, as in the case of the 2-naphthylalanine analogue, should increase the π electron density (Figure 5; 2-naphthylalanine) and strengthen the cation$-\pi$ interaction. Contrary to all these expectations, however, we did not observe a clear correlation between the π electron density and the activity or binding. In addition, the binding enthalpy remained unchanged when such analogues were incorporated. These observations strongly suggest that a cation$-\pi$ or $\pi-\pi$ interaction is not mediated by tryptophan at position 4 and is not required for the activity of compstatin.

It is also possible that incorporation of tryptophan analogues might change the structural features of compstatin and affect binding. However, we have previously reported that the substitution of valine with tryptophan and 2-naphthylalanine at position 4 on the structure of compstatin has negligible effect on its structure as examined by NMR.[11] In light of these observations, we concluded that it is not likely that the solution structure of compstatin changes upon substitution of tryptophan analogues or 2-naphthylalanine. However, these conclusions do not exclude the possibility of structural changes of bound form of compsatin, which we were attempting to obtain though cocrystallization experiments; the crystal structure of C3 was recently published.[26] Conformational changes upon compstatin binding to C3 have been previously suggested by use of surface plasmon resonance analysis.[27] In addition, the tryptophan side chain is believed to be facing outward as no interactions between tryptophan side chain and the rest of the molecule, which could stabilize the fold, were observed in the solution structure. This suggested that placing a fluorine atom or methyl group at position 5 of the indole ring might not affect compstatin's structure. Thus, we conclude that the log P versus IC_{50} and log P versus K_d correlations observed are due to the modulation of hydrophobic character of tryptophan, which in effect modulates the interaction of compstatin with C3. However, the same may not be true in case of compstatin analogue bearing 1-methyltryptophan due to its anomalous behavior (please refer to plots in Figure 4A,B). 1-Methyltryptophan has a methyl group attached to the indole nitrogen, unlike other analogues. This might place the methyl group in close proximity to the main chain of compstatin and affect the structure of compstatin in a way that it would bind to C3 with high affinity.

In summary, we propose that tryptophan at position 7 imparts the interaction through a hydrogen bond with the possibility of strengthening the interaction through incorporation of additional hydrogen-bond acceptors such as fluorine. In contrast, the tryptophan at position 4 prefers to interact through its hydrophobic nature, and the increase of hydrophobicity led to an increase in the activity. We suggest, on the basis of the results from the present study, that further optimization of the activity of compstatin can be made possible through introducing analogues that favor increase in the enthalpy change at position 7 and the analogues that favor entropy change at position 4.

Experimental Procedures

Reagents. Fmoc-5-fluoro-DL-tryptophan, Fmoc-5-methyl-DL-tryptophan, 2-naphthylalanine, and Fmoc-1-methyl-DL-tryptophan were obtained from Anaspec Inc. (San Jose, CA).

Peptide Synthesis and Purification. Analogues of compstatin containing 5-fluoro-L-tryptophan, 5-methyl-L-tryptophan, 1-methyl-L-tryptophan, L-tryptophan, or 2-naphthylalanine at positions 4 and/or 7 were synthesized chemically by solid-phase peptide chemistry according to standard protocols.[11] The N- and C-termini of the peptides were protected with acetyl and amide groups. All peptides were further purified on a C18 reversed-phase HPLC column to 95% purity, lyophilized, and characterized by MALDI mass spectrometry. For the synthesis of the analogues containing 1-methyltryptophan, 5-methyltryptophan, and 5-fluorotryptophan (Table 1), we used the Fmoc-DL-derivatives. Further separation of the diastereomeric peptides was performed as described by Meyers et al.[28] In brief, the DL mixture of each peptide was separated into D and L isomeric peptides on a C18 reversed-phase HPLC column with 10% acetonitrile in 0.01 M ammonium acetate, pH 4.1. The isomeric identity of the eluted peptides with incorporations at position 7 was determined by treating the peptides with V8 protease, followed by analysis by MALDI-TOF mass spectrometry (Micro-Mass TOFspec2E).

Purification of C3. C3 was purified from fresh human plasma obtained from the blood bank of the Hospital of the University of Pennsylvania as described previously.[29] Briefly, the plasma was fractionated with 15% (w/v) PEG 3350, and the pellet was resuspended in 20 mM phosphate buffer, pH 7.8, and then subjected to anion−exchange chromatography on a DEAE-HR 40, 50 × 5 cm column (Millipore Inc., Billerica, MA) with the same buffer. The proteins were eluted with 6 L of a linear gradient (15−70%) with20 mM phosphate buffer, pH 7.8, containing 500 mM NaCl. The C3 was further purified on a size-exclusion column Superdex 200 26/60 (Amersham Biosciences) and a Mono S column (Amersham Biosciences) to separate C3 from C3(H_2O).[27]

Inhibition of Complement Activation. Assays to assess the complement inhibitory activity of compstatin and its analogues were performed as described elsewhere.[11] In brief, they involved an

antigen−antibody complex-mediated complement activation of human serum, as detected by the deposition of C3b/iC3b to complex, in the presence of the compstatin analogue. The absorbance data obtained at 405 nm were translated into percent inhibition based on the absorbance corresponding to 100% complement activation. The percent inhibition was plotted against the peptide concentration, and the resulting data set was fit to the logistic dose−response function by use of Origin 7.0 software. IC_{50} values were obtained from the fitted parameters that achieved the lowest χ^2 value. Plots of IC_{50} versus log *P* were drawn by use of Origin 7.0 software. Log *P* values for tryptophan, 5-fluorotryptophan, 5-methyltryptophan, 1-methyltryptophan, and 2-naphthylalanine were calculated in JME molecular editor (www.molinspiration.com) from the structures shown in Figure 1.

Isothermal Titration Calorimetric Analysis of the Interaction of C3 with Compstatin and Its Analogues. Isothermal titration calorimetry experiments were performed with the Microcal VP-ITC calorimeter (Microcal Inc., Northampton, MA). Protein concentrations of 3.5−5 μM and peptide concentrations of 80−200 μM were used for these experiments. All titrations were performed in PBS (10 mM phosphate buffer with 150 mM NaCl, pH 7.4). In each experiment, the target protein, C3, was loaded into the cell, and peptide was loaded into the syringe. All experiments were performed at 25 °C, and for each experiment, 2-μL peptide injections were made into the cell containing the protein. In each experiment, the raw isotherms were corrected for the heats of dilution by subtracting the isotherms representing peptide injections into the buffer. The resulting isotherms were fit to various models by use of Origin 7.0 software, and the model that achieved the lowest χ^2 value was deemed to be appropriate for the respective data set. The binding affinity and entropy values were plotted against log *P* values by use of Origin 7.0 software.

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