Compstatin is a 13-residue cyclic peptide that inhibits complement activation by binding to complement component, C3. Although the activity of compstatin has been improved severalfold using combinatorial and rational design approaches, the molecular basis for its interaction with C3 is not yet fully understood. In the present study, isothermal titration calorimetry was employed to dissect the molecular forces that govern the interaction of compstatin with C3 using four different compstatin analogs. Our studies indicate that the C3-compstatin interaction is an enthalpy-driven process. Substitution of the valine and histidine residues at positions 4 and 9 with tryptophan and alanine, respectively, resulted in the increase of enthalpy of the interaction, thereby increasing the binding affinity for C3. The data also suggest that the interaction is mediated by water molecules. These interfacial water molecules could be the source for unfavorable entropy and large negative heat capacity changes observed in the interaction. Although part of the negative heat capacity changes could be accounted for by the water molecules, the rest might be resulting from the conformational changes in C3 and/or compstatin up on binding. Finally, we propose based on the pKₐ values determined from the protonation studies that histidine on compstatin participates in protonation changes and contributes to the specificity of the interaction between compstatin and C3. These protonation changes vary significantly between the binding of different compstatin analogs to C3.

The complement system is a distinctive arm of the innate immune system that plays a critical role in the immune response. It is known to be activated through at least three different pathways, all of which converge at a single point involving the complement component C3. Numerous disease states have been described in which an overactive complement system has been implicated (1, 2), and in such situations complement needs to be inhibited. To date, several inhibitors have been designed to inhibit the complement system at several points in the cascade. The first inhibitor ever capable of inhibiting all three pathways of complement was the 13-amino acid cyclic peptide compstatin, identified by our group (3). This peptide, which blocks the complement cascade by binding to C3, was identified by screening a phage-display library. Compstatin exhibits a higher activity when its N terminus is acetylated and C terminus is amidated.

In an attempt to understand the structure-activity relationships involved in the interaction between compstatin and C3, the three-dimensional structure of the free peptide in solution was previously determined using NMR (4). The results indicated that in solution the peptide assumes a coil conformation with a type 1 β-turn. Alanine scanning revealed that the β-turn, comprising residues Gln-5, Asp-6, Trp-7, and Gly-8, is critical for activity of the peptide, and this turn has, therefore, been regarded as an important structural determinant of compstatin activity (4). In contrast, alanine scanning of residues outside the β-turn yielded a more potent peptide in which His-9 was substituted with alanine, whereas substitution of other residues with alanine lowered the activity (4, 5).

Several efforts have been made to improve the inhibitory activity of compstatin using computational techniques (6), rational design (5), and phage-display library screening. In addition, global optimization studies carried out on this peptide indicated that an analog in which Val-4 was substituted with tyrosine was 16 times more potent (6). Guided by these modifications, further substitutions were targeted at position 4 using several aromatic amino acids; these studies identified a peptide with a tryptophan at position 4 and alanine at position 9 that showed a 45-fold higher potency when compared with the initial identified peptide. This peptide is referred here as V4W/H9A. Despite all these improvements made in the activity of the peptide, the binding mechanism of compstatin to C3 remains elusive.

Surface plasmon resonance (SPR) studies of the C3-compstatin interaction suggested that compstatin binds to C3 with a 1:1 stoichiometry (7). Although these studies explained the kinetics of the interaction, no information about the nature of the interaction was deduced. Therefore, the present study using isothermal titration calorimetry (ITC) was designed to yield insight into the nature of the interaction between C3 and compstatin. ITC, unlike SPR, permits the study of macromolecular interactions in solution. Using four different compstatin analogs, we were able to dissect the molecular interactions that define the association of compstatin with C3. The results of this study led to the formulation of a working molecular model for C3-compstatin interaction.

EXPERIMENTAL PROCEDURES

Purification of Proteins—C3 was purified from fresh human plasma obtained from the blood bank at the Hospital of The University of
Thermodynamics of the C3-Compstatin Interaction

RESULTS

Energetics of the C3-Compstatin Interaction—To study the thermodynamics of the C3-compstatin interaction, four different analogs designated V4H9, H9A, V4W, and V4W/H9A were used in the ITC experiments. The binding isotherms representing the binding events fit to a single set of binding sites model, suggesting an equimolar binding (Fig. 1). The four peptides bound to C3 in the increasing order of their affinities as follows: V4H9 (Kd = 1.3 μM) > H9A (Kd = 0.9 μM) > V4W (Kd = 0.36 μM) > V4W/H9A (Kd = 0.14 μM). Comparing these values with the activity3 of these peptides observed previously indicate that there exists a direct linear correlation between the binding and activity. In other words, binding of compstatin analogs to C3 proportionally affects their activity (data not shown).

Our experiments indicate that the interaction between C3 and compstatin is characterized by unfavorable positive entropy and favorable negative enthalpy changes, indicating an enthalpy-driven process (Tables I and II). Isotherms representing the interaction, corrected for heats of dilution are shown in Fig. 1, A–D. As shown in the figure, the data fit to a 1:1 single set of binding sites model, which yielded all the thermodynamic parameters. Although the binding of the parent peptide V4H9 to C3 was characterized by a −12.12 kcal/mol enthalpy change and a −8.08 kcal/mol free energy change, the binding of V4W/H9A involved a −18.14 kcal/mol enthalpy change and a −9.4 kcal/mol free energy change. The enthalpy gain observed after substitution of positions 4 and 9 with tryptophan and alanine can most likely be attributed to additional polar non-covalent interactions initiated by these residues.

To dissect the factors that dictate the enthalpy and entropic differences observed between the binding of V4H9 and V4W/H9A to C3, we tested the analogs, V4W and H9A, in ITC studies. Substitution of histidine at position 9 with alanine (H9A) increased the ΔH of the interaction by −2.2 kcal/mol (refer to ΔΔH, Fig. 2). On the other hand, substitution of valine at position 4 with tryptophan (V4W) also increased the enthalpy by −2.5 kcal/mol (ΔΔH, Fig. 2). These results indicate that tryptophan and alanine contribute equally to the binding enthalpy of the C3-V4W/H9A interaction. In addition, studies carried out with H9A and V4W indicated that tryptophan and alanine contribute equally (ΔΔΔS, Fig. 2) to the entropic penalty of binding and that the individual contributions add up to the total entropy loss suffered by V4W/H9A upon binding to C3.

Effect of Temperature on Binding—In several protein-protein and protein-peptide interactions, calorimetric enthalpy could be affected by changes in the experimental temperature. Therefore, the calorimetric titrations were performed over a temperature range of 15–37 °C. Fig. 3 shows the calorimetric enthalpy, entropy, and free energy changes plotted against temperature. The enthalpy and entropy changes in the binding of V4H9 and V4W/H9A to C3 showed strong temperature dependence. In addition, a linear correlation between the enthalpy and entropy (Fig. 4) was observed, which is a common feature in macromolecular interactions. Such dependence has previously been shown in several studies involving macromolecular interactions (9–13). Although the enthalpy change increased, the entropy change decreased in a linear fashion with an increase in temperature. However, the free energy change remained unaffected. A linear dependence of calorimetric enthalpy on temperature indicates a two-state binding process with equilibrium between the free and bound forms. In contrast, a non-linear relationship indicates a complex process of binding.

Temperature dependence of the enthalpy of binding can lead...
to heat capacity changes ($\Delta C_p$) upon binding, according to the relationship in Equation 3.

$$\Delta C_p = \left(\frac{\partial \Delta H}{\partial T}\right)_p$$  \hspace{1cm} (Eq. 3)

The slope of the plot of $\Delta H$ versus temperature yields $\Delta C_p$. As shown in Fig. 3, the plot of $\Delta H$ versus temperature was linear and yielded a straight line with a correlation value of 0.99 for V4W/H9A and 0.98 for V4H9 binding. $\Delta C_p$ was calculated from the slope of the plots to be $-413$ cal/mol/K for C3-(V4W/H9A) binding and $-194$ cal/mol/K for C3-(V4H9) binding.

**Effect of Buffer Ionization and pH on the C3-Compstatin Interaction**—Often the enthalpy observed in a calorimeter is a result of the binding event in addition to all the associated events (water a(di)ssociation, ionization of the components, heats of dilution, heats of mixing, etc). To facilitate binding, residues at the interface may be protonated or deprotonated, resulting in exchange of protons with the buffer. Under such circumstances, calorimetric enthalpy is dependent on the buffer ionization enthalpy. To study the dependence of buffer ionization on calorimetric enthalpy, ITC titrations were performed in phosphate, Tris-HCl, and MOPS buffers at pH 7.4.
An increase in the enthalpy change ($\Delta H_{\text{obs}}$) was observed with an increase in the buffer ionization enthalpy change ($\Delta H_{\text{in}}$) when V4H9 bound to C3. In contrast, when V4W/H9A bound to C3, a decrease in the enthalpy change was observed with an increase in the buffer ionization enthalpy change. A plot of calorimetric enthalpy against ionization enthalpy yielded the number of protons ($n_{H^+}$) involved in the interaction, and the binding enthalpy was corrected for protonation effects ($\Delta H_{\text{bio}}$) according to the following relationship:

$$\Delta H_{\text{obs}} = \Delta H_{\text{in}} + n_{H^+} \Delta H_{\text{in}}$$  \hspace{1cm} (Eq. 4)

Plots shown in Fig. 5, A and B, yielded an $n_{H^+}$ value of −0.35 and a binding enthalpy ($\Delta H_{\text{in}}$) of −12.32 kcal/mol for the C3-(V4H9) interaction and values of +0.31 and −18.05 kcal/mol for the C3-(V4W/H9A) interaction. Although a positive slope indicates propensity for uptake of protons from the buffer, a negative value indicates propensity for release of protons into the buffer. Thus, the interaction between C3 and V4H9 appeared to be mediated by a net release of protons into the buffer, in contrast to the interaction between C3 and V4W/H9A, which involved a net uptake of protons from the buffer.

In addition to the buffer effects, experimental pH can have a pronounced influence on the ionization of residues because of changes in their $pK_a$ values. To determine the effect of pH on the interaction between compstatin and C3, we carried out ITC studies at pH 6.7. Values of pH lower than 6.7 were not employed because of the reduced stability of C3 at lower pH. In these experiments, both peptides V4H9 and V4W/H9A exhibited calorimetric enthalpies lower than those at pH 7.4 (Fig. 5, A and B). However, the effect of buffer ionization on the observed calorimetric enthalpies was similar to that observed at pH 7.4. Plots of $\Delta H_{\text{obs}}$ versus $\Delta H_{\text{in}}$ at pH 6.7 gave $n_{H^+}$ and $\Delta H_{\text{in}}$ values of −0.1 and −12.23 kcal/mol for the C3-V4H9 interaction and 0.47 and 18.2 kcal/mol for the C3-V4W/H9A interaction. The number of protons released from or taken up at the interface upon binding of compstatin to C3 indicates in the simplest case a single residue as responsible for the observed protonation changes. The contrasting protonation changes observed between binding of V4H9 and V4W/H9A can explain the effect of the amino acid modifications on the selectivity of compstatin in molecular recognition.

Clearly, the number of protons involved at the interface showed a significant dependence on the experimental pH (Fig. 5, A and B). Baker and Murphy have proposed that a change in the number of protons released or taken up at the interface upon binding depends on the proton binding constants and experimental pH according to the following relationship (14).
This relationship holds only when a single amino acid residue is assumed to be involved in protonation or deprotonation at the binding interface,

\[
\begin{align*}
\Delta H &= K_p^c \cdot 10^{-\Delta pH} - K_p^f \cdot 10^{-\Delta pH} \\
\Delta G &= 0.055 \cdot \log(\text{solute osmol})
\end{align*}
\]  

(Eq. 5)

where \(K_p^c\) and \(K_p^f\) are the proton binding constants for the free and complexed forms. The binding constant is equal to \(10^{\Delta pK_a}\) of the ionizing group. In principle, the determination of \(n_H\) at two different \(pH\) values should suffice to calculate the \(pK_a\) of the ionizing group. Based on the \(n_H\) values obtained at \(pH\) 7.4 and 6.7, \(pK_a^c\) and \(pK_a^f\) were calculated to be 8.97 and 7.62 for the C3-V4H9 and 6.3 and 7.2 for the C3-V4W/H9A interactions. These \(pK_a\) changes between free and bound forms are consistent with the release of protons observed when V4H9 bound to C3 and with the uptake of protons when V4W/H9A bound to C3.

**Effect of Solvent on the C3-Compstatin Interaction**—Because water serves as a solvent for most biological processes, it is very important to understand the biomolecular interactions that occur in the presence of water molecules. In the case of protein-protein interactions, water molecules act as molecular determinants of ligand recognition, thus altering the specificity of the interaction (15–17). To gain insight into the role of water molecules in the molecular recognition of compstatin by C3, we carried out osmotic stress experiments. In the present study, glycerol was included in the various buffers at different concentrations to induce osmotic stress. Glycerol molecules, because of their large size relative to water, are excluded from water-filled spaces at the binding interface, thus changing the activity of the bulk solvent. The subtle changes in the free energy that occur when the water activity is altered as a result of osmotic stress are indicators of differences in the hydration of complexed and uncomplexed states.

For both the C3-V4H9 and C3-V4W/H9A interactions, differences in the free energy changes were observed in response to changes in solute osmolality (Tables I and II), consistent with the exclusion of solute molecules from the binding interface between C3 and compstatin. Also, a decrease in the association constant was observed with an increase in osmolality (Fig. 6). This increase indicates that osmotic stress weakens the interaction between C3 and compstatin. Based on the water activity and solute osmolality, the following relationship can be used to calculate the change in the number of solute-excluded water molecules (\(\Delta n_w\)) associated with the binding event (17).

\[
\frac{\Delta \log K_p}{\Delta \text{osmol}} = \frac{-2.303 \cdot \Delta n_w}{\text{osmol}}
\]  

(Eq. 6)

Plotting the logarithm of the association constant (\(K_p\)) against solute osmolality resulted in a slope of ~0.17 for V4H9 and ~0.12 for V4W/H9A binding. These slopes were further translated into \(\Delta n_w\) using the above equation, yielding values of 4 for the C3-V4H9 and 3 for the C3-V4W/H9A interactions. Although a positive value for \(\Delta n_w\) indicates uptake of water molecules at the vicinity of the binding interface, a negative value indicates concomitant release of water molecules. Thus, binding of V4H9 to C3 was accompanied by uptake of four water molecules at the interface, whereas binding of V4W/H9A to C3 was associated with the uptake of three water molecules. This type of interaction is in contrast to the release of water molecules observed in the formation of most protein-protein and protein-peptide complexes (17–19).
In the present study ITC was employed to elucidate the interaction of compstatin with C3. In an effort to explain the increase in the binding activity of compstatin observed after substitutions at positions 4 and 9, four different compstatin analogs were used to pursue this goal. All the four analogs bind to C3 with different affinities, which reflect the differing activities of these analogs. However, the dissociation constants obtained using calorimetry for the interaction of V4H9 with C3 varied considerably from those obtained using Biacore (7) and from inhibitory constants (concentration at 50% inhibition) obtained by enzyme-linked immunosorbent assay.1 Similar differences have previously been observed in the case of the interaction between cyclophilin and cyclosporin A (20, 21). These differences can presumably be attributed to the differing sensitivities of these techniques to the various aspects of complex formation. For example, differences between ITC and SPR data have been very commonly observed in other studies involving proteins and peptides (22). Such variations were attributed to possible differences in the behavior of the proteins when immobilized on the chip and in solution. It is also possible that these discrepancies arise from improper analysis of the SPR data.

The interaction between C3 and compstatin was characterized by favorable enthalpy and unfavorable entropy changes. Although a favorable enthalpy change suggests the prevalence of several polar non-covalent electronic interactions, including main-chain and side-chain hydrogen bonds and salt bridges, an unfavorable entropy change suggests a loss of degrees of freedom upon binding. It should be noted that V4W/H9A bound to C3 with at least 6 kcal/mol higher enthalpy than did V4H9 (Tables I and II), suggesting an increase in favorable non-covalent interactions at the binding interface upon substitution at positions 4 and 9. In contrast, an increased entropy loss was observed with V4W/H9A binding when compared with V4H9 binding, in effect increasing the free energy of binding (Tables I and II). More favorable non-covalent interactions and the free energy of binding explain the higher binding affinity observed for V4W/H9A to C3.

The differences in enthalpy change observed between V4H9 and V4W/H9A binding might arise from changes in the protonation or deprotonation of some residues at the interface. The results of our protonation experiments suggested that the C3-V4H9 interaction is associated with deprotonation of one residue, in contrast to C3-V4W/H9A interaction, which involved protonation at the interface. These observations were further supported by the pK_a values calculated for this residue before and after binding in each of these interactions. The pK_a values calculated in the present study are in good accordance with values observed for histidines involved in catalysis (23–27) and macromolecular complex formation (28, 29), which makes histidine the most likely candidate to be assigned for the protonation changes at the C3-compstatin interface. V4H9 has two histidines at positions 9 and 10, whereas V4W/H9A has only one histidine at position 10, and the other histidine at position 9 is replaced by an uncharged alanine residue. Thus, it is possible that the protonation changes observed between the interactions of two peptides with C3 can be attributed to the histidines at positions 9 and 10. Alternatively, it is also possible that a residue (most likely histidine) on C3 becomes charged through protonation and interacts with other charged residues or tryptophan on V4W/H9A. To test this hypothesis, we studied the interaction of the H9A analog with C3 in various buffer systems at pH 7.4, studies identical to those carried out with V4H9 and V4W/H9A. As we saw with the other two peptides, this analysis showed a linear dependence of calorimetric enthalpy on the buffer ionization enthalpies. The calculated fit yielded an n_H^+ value of +0.28 and a binding enthalpy of -14.6 kcal/mol (Fig. 5C). Interestingly, the sign and magnitude of n_H^+ were similar to those observed for the interaction between V4W/H9A and C3, confirming that the alanine substitution is probably responsible for the protonation changes. Alanine may not be directly involved in protonation changes because it lacks ionizable side chain. Therefore, consistent with the pK_a values calculated for the C3-(V4W/H9A) interaction, it is possible that alanine changes the dielectric conditions surrounding His-10, thus allowing it to be protonated.

Based on these observations and the pK_a values calculated for the C3-V4H9 and C3-V4W/H9A interactions, we hypothesize that His-9 on V4H9 is protonated before binding and...
releases a proton upon binding, whereas His-10 remains neutral both before and during binding. In contrast, His-10 in V4W/H9A is neutral before binding and shows propensity for protonation upon binding, thus facilitating an additional enthalpically favorable interaction ($\Delta \Delta H = -2.2$ kcal/mol, Fig. 2).

The protonated histidine might interact with C3 in three possible ways: salt-bridge formation with a negatively charged residue on C3, a cation-π interaction with an aromatic residue on C3, or by forming a charged hydrogen bond with a hydrogen bond acceptor on C3.

Unlike the possible indirect contribution showed by alanine to the enthalpy gain observed during the C3-V4W/H9A interaction, tryptophan can also potentially contribute directly to this enthalpy gain. This possibility was supported by the enthalpy gain of $-2.5$ kcal/mol observed in the C3-V4W interaction (refer to $\Delta \Delta H$, Fig. 2). Studies involving substitution of valine at position 4 with various natural and un-natural aromatic amino acid analogs that differ in ring conjugation have suggested that an aromatic amino acid at this position could be involved in a cation-π interaction with either lysine or arginine on C3. Consistent with these observations, an increase in enthalpy was observed upon substitution of valine with tryptophan. This finding indicates that the cation-π interaction between tryptophan at position 4 of compstatin and either lysine or arginine on C3 contributes an enthalpy of $-2.5$ kcal/mol ($\Delta \Delta H$, Fig. 2) and a free energy change of $-0.8$ kcal/mol to the binding ($\Delta \Delta G$, Fig. 2).

The energetic parameters obtained for C3-V4H9 and C3-V4W/H9A showed a strong dependence on experimental temperature. Despite the differences observed in enthalpy and entropy change with temperature, the free energy change remained minimal, suggesting enthalpy-entropy compensation. This phenomenon is evident from the slope of the line fit to the plot of $\Delta H$ and $T \Delta S$ (Fig. 4); a slope close to 1 indicates enthalpy-entropy compensation. Enthalpy-entropy compensation has been a general feature of the weak molecular interactions that undergo constant rearrangements to realize a lower free energy of binding. Enthalpy-entropy compensation has been observed in most protein-protein and protein-peptide interactions (30–34). In addition, the temperature dependence of calorimetric enthalpy revealed a large negative heat capacity change during the binding of V4H9 and V4W/H9A to C3. Large changes in negative heat capacity change indicate a reduction in the nonpolar solvent-accessible surface area, as explained by the equation (35),

$$\Delta C_p = 0.45(\Delta \Delta \text{ASA}_{\text{nonpol}}) - 0.26(\Delta \Delta \text{ASA}_{\text{pol}}) \text{cal/mol K} \quad \text{(Eq. 7)}$$

where $\Delta \Delta \text{ASA}_{\text{nonpol}}$ is the change in the non-polar-accessible surface area, and $\Delta \Delta \text{ASA}_{\text{pol}}$ is the change in the polar-accessible
surface area. Such large negative heat capacity changes have been observed before in other protein-peptide interactions, in protein folding governed by hydrophobic effect (36, 37), and in macromolecular complex formation associated with the burial of solvent-exposed hydrophobic residues (10, 11, 38–40).

A large negative heat capacity of binding can also arise from the binding of water molecules at the interface, as has been shown in several instances (10, 15, 16, 46). Water molecules have been commonly found at the binding interface, as shown in several X-ray crystal structures of protein-protein and protein-peptide complexes. These water molecules can act as molecular bridges mediating interactions between proteins and ligands through hydrogen bonds (15, 47) or by occupying void spaces, thus changing the shape complementarities between the protein and ligand surfaces (48, 49). In the present study we showed that C3-compstatin interaction is mediated by water molecules. These water molecules may stabilize local hydrogen bonding networks and (or) affect pK_a values. However, in the absence of the crystal structure for the C3-compstatin complex, it is not possible to determine how water molecules contribute to the interaction between C3 and compstatin.

It was proposed on the basis of studies of novobiocin binding to DNA gyrase that sequestration of one water molecule at the binding interface reduces the heat capacity change by 48 cal/mol/K (46). Taking this relationship into account, we can estimate the heat capacity change produced by water molecules in our binding reactions to be –192 cal/mol/K (ΔC_p = –194 cal/mol/K) for C3-V4H9 and –144 cal/mol/K (ΔC_p = –413 cal/mol/K) for C3-V4W/H9A. Based on the heat capacity changes calculated from the temperature dependence of the calorimetric enthalpy, all the heat capacity change observed during C3-V4H9 binding can be attributed to four water molecules sequestered at the binding interface. In contrast, only part of the heat capacity change (i.e. –144 cal/mol/K) is accounted for by the three water molecules sequestered at the binding interface of V4W/H9A and C3. Therefore, the rest (–269 cal/mol/K) can be attributed to the burial of solvent-exposed hydrophobic residues or any conformational changes.

In the classical picture of the hydrophobic effect, burial of solvent-exposed residues, which gives rise to negative heat capacity changes, accompanies reorganization of the solvent molecules, thus increasing solvation entropy (41). This process contradicts the unfavorable entropy changes observed during C3-compstatin interaction. Thus, the unfavorable entropy changes we observed could be due to possible conformational changes occurring in C3 and/or compstatin upon binding and/or to the binding of water molecules at the interface of the interacting molecules. Studies have been reported in which unfavorable entropy changes were ascribed to configurational changes in the ligand upon binding (42) or to protein conformational changes upon binding (43). Molecular dynamic simulations carried out on the ensemble of energy-minimized NMR structures suggested that V4H9 exists as multiple conformers in solution (44). The authors reported five major conformers of compstatin in solution: The most populated is the coil conformation with a type I β-turn occupying about 44% of the conformational space, which resembles the average energy minimized conformation obtained from NMR studies. The second and third most populated are β-hairpins with type I or type II β-turns, occupying about 39% of the conformational space. Based on these observations and the negative heat capacity changes observed in the present study, we propose that compstatin exists as a coil before binding and shows a propensity to undergo configurational changes to a more structured β-hairpin after binding. This interconversion has a low free energy barrier, showing a higher probability for the peptide to assume a β-hairpin conformation from the coil conformation (44).

NMR studies of the V4W/H9A analog have suggested that this peptide, like V4H9 and H9A, assumes a coil conformation with a β-turn when free in solution.1 Therefore, any changes in the energetics observed between C3-V4H9 and C3-V4W/H9A binding could be a result of additional direct interactions by tryptophan at position 4 on V4W/H9A, as previously mentioned. It should be noted that additional intramolecular interactions are possible within compstatin if we assume that compstatin acquires a more ordered β-hairpin conformation upon binding. The enthalpy arising from these interactions could make a contribution to the net enthalpy changes in binding. Because there is no three-dimensional structure information available for C3, we could not make any definitive conclusions as to whether any conformational changes occur in C3 upon binding. However, it is possible that the peptide recognition region on C3 is flexible before binding and that compstatin binding stabilizes the interface in a more fixed configuration, as has been shown for cyclosporin A binding to human cyclophilin 18 (43). Such a conformational entropy penalty of binding has also been observed in the case of T cell receptor binding to peptide-major histocompatibility complex (45). In addition to the contributions from the solvent and conformational changes, the entropic term includes contributions from the rotational and translational degrees of freedom. However, from our data it is not possible to assess the entropic costs of these elements.

In summary, this study demonstrates detailed understanding of the thermodynamics of the C3-compstatin interaction, emphasizing the effects of key residue substitutions on compstatin on the interaction. Briefly, substitution of tryptophan at position 4 and alanine at position 9 of compstatin allows additional enthalpically favorable non-covalent interactions, thus increasing the affinity of the interaction. It is plausible to suggest that substitution of alanine at position 9 changes the ionization state of the histidine at position 10, thus changing the specificity of the interaction. In addition, it was shown that water molecules mediate the interaction, thus adding an unfavorable entropic term to the energetics of the process.

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