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Thermodynamic and Structural Effects of Macrocyclic Constraints in Protein−Ligand Interactions

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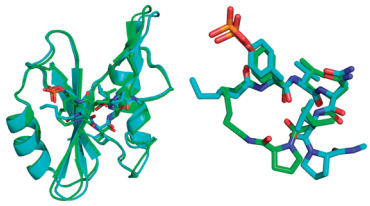
ABSTRACT The thermodynamic and structural effects of macrocyclization as a tactic for stabilizing the biologically active conformation of Grb2 SH2 binding peptides were investigated using isothermal titration calorimetry and X-ray crystallography. 23-Membered macrocycles containing the sequence pYVN were slightly more potent than their linear controls; however, preorganization did not necessarily eventuate in a more favorable binding entropy. Structures of complexes of macrocycle **7** and its acyclic control **8** are similar except for differences in relative orientations of corresponding atoms in the linking moieties of **7** and **8**. There are no differences in the number of direct or water-mediated protein−ligand contacts that might account for the less favorable binding enthalpy of **7**; however, an intramolecular hydrogen bond between the pY and the pY+3 residues in **8** that is absent in **7** may be a factor. These studies highlight the difficulties associated with correlating energetics and structure in protein−ligand interactions.

KEYWORDS Protein−ligand interactions, preorganization, macrocyclic peptides, x-ray crystallography, isothermal titration calorimetry



The design of molecules that bind to proteins with high affinity is one of the primary objectives in medicinal chemistry. Toward this end, one common tactic for increasing ligand binding affinities involves preorganizing ligands into their biologically active conformations by introducing conformational constraints.[1] When peptides serve as starting points for such investigations, preorganization is typically achieved by introducing rings via bond formation between a side chain and a backbone atom, between two side chains, or between the N and the C termini of the peptide. The rationale for this approach owes its origin to the conventional wisdom that binding of a preorganized ligand should be entropically favored over its more flexible counterpart because of a reduced conformational entropy that is associated with restricting rotors.[2] Provided that the two ligands interact in the same way with solvent and the protein (i.e., $\Delta\Delta H° \sim 0$ kcal mol^{-1}), the preorganized molecule would thus be expected to benefit from a more favorable binding free energy. However, in studies of the binding of phosphotyrosine-derived peptides to Src and Grb2 SH2 domains, we have discovered that ligand preorganization can have either favorable or unfavorable entropic consequences.[3−5] In the general context of our interest in energetics and structure in protein−ligand interactions, we now report energetic and structural effects of introducing macrocyclic conformational constraints to preorganize Grb2 SH2 binding ligands.

The Grb2 SH2 domain binds peptides containing the amino acid sequence pTyr-Xaa-Asn (pYXN) in a type 1 β-turn conformation that is stabilized by an intramolecular hydrogen bond between the carbonyl oxygen atom of the pY residue and the backbone amide nitrogen atom of the pY+3 residue.[6] It has been shown that macrocyclization of a phosphotyrosine-derived peptide containing the pYVN sequence can enforce such a turned structure in solution, whereas the corresponding linear control adopts a random coil conformation.[7] A number of cyclic ligands having ring sizes varying from 14[8] to 33[9] atoms have subsequently been shown to bind to the Grb2 SH2 domain with high affinity. Because the binding entropies and enthalpies of these macrocycles and their linear controls were not determined, the detailed energetic consequences of ligand preorganization by macrocyclization in these systems are unknown. Indeed, there is but a singular such study that was reported by Spaller and coworkers who compared binding enthalpies and entropies for the complex formation of macrocyclic ligands and their linear controls with the PDZ3 domain of PSD-95.[10]

To define explicitly the effects of macrocyclization as a tactic for preorganizing Grb2 SH2 binding ligands, it is necessary to compare the binding energetics and structures of complexes of constrained and flexible ligand pairs having the same number and type of nonhydrogen atoms, the same number of hydrogen bond donors and acceptors, and the same functional groups. We initiated our studies with the 20-membered macrocycles **1** and **2**, which contain the amino acid sequences pYVNG and pYVNV, respectively, and their

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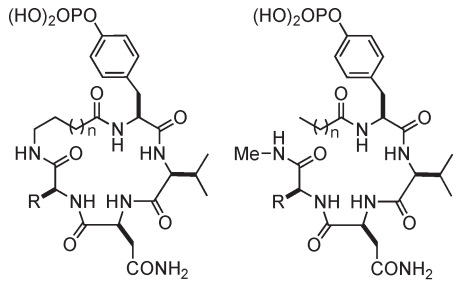
corresponding acyclic controls **4** and **5**. The valine derivatives **2** and **5** were of interest because several structural studies suggest that the valine side chain at the pY+3 site forms favorable van der Waals contacts with the Grb2 SH2 domain.[6,11] The acyclic controls **4** and **5** and the linear precursors of **1** and **2** were synthesized by straightforward peptide coupling procedures, and the macrocycles **1** and **2** were prepared by cyclization of their respective α,ω-amino acid precursors using pentafluorophenyl diphenylphosphinate.[12]



1: n = 4, R = H
2: n = 4, R = $CHMe_2$
3: n = 7, R = H

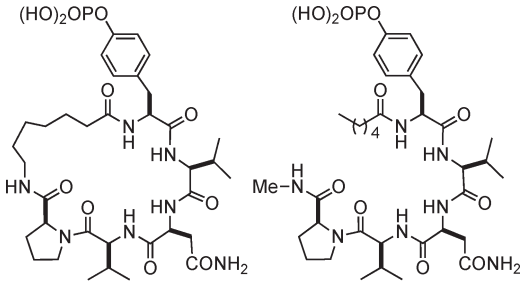
4: n = 4, R = H
5: n = 4, R = $CHMe_2$
6: n = 7, R = H

The thermodynamic parameters (K_a, $\Delta G°$, $\Delta H°$, and $\Delta S°$) for binding of the pairs of macrocyclic and linear peptides **1/4** and **2/5** to the Grb2 SH2 domain were determined using isothermal titration calorimetry (ITC) as described previously (Table 1).[4] The macrocycle **1** bound with about 10-fold lower affinity than its acyclic control **4**, primarily because of a much less favorable binding enthalpy for **1**. Compounds **2** and **5** each bound with approximately the same affinity, and the binding enthalpies and entropies for the two ligands were comparable. Incorporation of a valine residue at the pY+3 site did indeed confer the expected higher affinity binding with the association constants for **2** and **5** being approximately 50- and 6-fold greater than **1** and **4**, respectively.

We undertook structural studies of complexes of these ligands with the Grb2 SH2 domain but were only able to obtain diffractable crystals of the complex of **2**. The structure of the complex of **2** was solved to 2.0 Å resolution, and there are three coexisting complexes in the asymmetric unit. Analysis of these complexes reveals that the pY-1 carbonyl oxygen atom of **2** makes a polar contact with the guanidinium moiety of the domain residue Arg67 in only one of the three complexes. This finding was wholly unexpected because this carbonyl oxygen atom is involved in two direct polar contacts with the side chain of Arg67 in all known complexes of phosphotyrosine-derived ligands bound to the Grb2 SH2 domain.

On the basis of the foregoing observations, we reasoned that a 20-membered ring in cyclic analogues of pYVNG(V) might not be sufficiently large to allow for optimal protein−ligand interactions and consequently shifted our attention to larger rings containing an additional amino acid residue. The 23-membered macrocycle **3** and the corresponding linear control **6** were synthesized, and the thermodynamic parameters for binding of **3** and **6** to the Grb2 SH2 domain were determined by ITC as before (Table 1). The macrocycle **3** bound to the domain approximately 20-fold better than its 20-membered-ring analogue **1**, and it exhibited comparable, albeit slightly greater, affinity for the domain than its linear control **6**.

We were equally interested in studying the 23-membered analogues of **2** and **5** to further assess the energetics associated with incorporating a valine residue at the pY+3 site, but the insolubilities of these compounds prevented us from obtaining ITC data. Accordingly, we turned our attention to the pYVNVP-derived macrocycle **7** and its linear control **8**. We were inspired toward this end by the work of Ettmayer and co-workers,[7] who had shown in a bioassay that **7** was about 3-fold more potent than a linear control closely related to **8**. ITC studies of **7** and **8** reveal that **7** was significantly more potent than any other ligand in this study and bound with about 2-fold greater affinity than **8** (Table 1).



7 **8**

A comparison of the binding energetics for the two 23-membered macrocycles **3** and **7** with their corresponding acyclic controls reveals an intriguing fact. Namely, the small increase in binding affinity of **3** over its more flexible linear control **6** arises from a relatively more favorable enthalpy of binding that overrides a compensating entropic term that is less favorable for **3** than for **6**. On the other hand, the slightly enhanced affinity of **7** relative to **8** eventuates because a more favorable entropic term for binding of **7** dominates a compensating enthalpy term that is less favorable than for **8**. On the basis of these observations, it is clear that preorganization of pYVN-derived ligands by macrocyclization can have either a favorable or an unfavorable entropic effect. The variable entropic effects of preorganization of these ligands may arise from differences in the nature and flexibility of the linking moiety, which does not interact directly with the domain itself, which connects the N and the C termini. Such an interdependence of binding energetics upon linker structure has also been observed by Spaller and co-workers in their studies of macrocyclic and linear ligands that bind to the PDZ3 domain.[10] However, contributions associated with varying the pY+4 residue from glycine to valine in the two sets of ligands cannot be excluded.

One must exercise caution in interpreting the results from these thermodynamic studies because the differences in $\Delta G°$ for the ligands being compared are small. Moreover, we do not understand the underlying basis for the observed fluctuations in the compensating enthalpies and entropies for binding of the respective ligand pairs. For example, **4** and **6**, whose structures differ by three methylene groups, bind with comparable affinities even though their binding enthalpies and entropies vary considerably. Because the

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Table 1. Thermodynamic Data for Binding of Macrocyclic and Acyclic Phosphotyrosine-Containing Peptides to the Grb2 SH2 Domain Obtained by ITC at 298 K[a]

ligand	K_a (M^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$\Delta S°$ (cal mol^{-1} K^{-1})	$-T\Delta S°$ (kcal mol^{-1})
1	$(4.7 \pm 0.11) \times 10^4$	-6.4 ± 0.01	-3.5 ± 0.26	9.8 ± 0.20	-2.9 ± 0.06
4	$(4.1 \pm 0.17) \times 10^5$	-7.7 ± 0.03	-6.3 ± 0.40	4.6 ± 0.35	-1.4 ± 0.10
2	$(2.3 \pm 0.21) \times 10^6$	-8.7 ± 0.06	-4.3 ± 0.32	14.8 ± 0.54	-4.4 ± 0.16
5	$(2.3 \pm 0.12) \times 10^6$	-8.7 ± 0.03	-4.6 ± 0.23	13.6 ± 0.44	-4.1 ± 0.13
3	$(8.5 \pm 0.03) \times 10^5$	-8.1 ± 0.03	-6.3 ± 0.68	5.9 ± 0.37	-1.8 ± 0.12
6	$(5.7 \pm 0.65) \times 10^5$	-7.9 ± 0.07	-4.8 ± 0.44	10.2 ± 1.00	-3.1 ± 0.32
7	$(1.0 \pm 0.20) \times 10^7$	-9.6 ± 0.09	-4.3 ± 0.57	17.7 ± 1.20	-5.3 ± 0.36
8	$(6.5 \pm 0.13) \times 10^6$	-9.3 ± 0.01	-6.3 ± 0.38	9.9 ± 0.17	-3.0 ± 0.06

[a] At least two experiments were performed for each ligand, and the averages are reported; errors are determined as previously reported.[4]

magnitudes of variations in binding energetics of these two linear ligands are not too dissimilar from those for the two constrained/flexible ligand pairs **3**/**6** and **7**/**8**, the significance of the observed entropic and enthalpic differences relevant to preorganization of **6** and **8** is difficult to evaluate.

The thermodynamic parameter ΔC_p, which is the temperature dependence of $\Delta H°$, is often correlated in biological systems with the burial of nonpolar and polar surfaces. ΔC_p values are thus frequently compared as a means of ascertaining whether desolvation effects might be involved in differential binding energetics.[13] The ΔC_p values for **3** and **6**−**8** were thus determined and found to be -158.1 ± 7.7, -199.8 ± 5.2, -222.3 ± 3.2, and -239.0 ± 13.5 cal mol^{-1} K^{-1}, respectively.[14] The differences in ΔC_p for the constrained/flexible pair **3** and **6** are thus significant relative to experimental error, but ΔC_p values for **7** and **8** are only slightly outside experimental error. Hence, dissimilarities in the thermodynamics of binding of **3** and **6** might result partly from differences in desolvation, whereas desolvation appears to play little role in the differential binding energetics of **7** and **8**.

The three-dimensional structures of the Grb2 SH2 domain complexed with **7** and **8** were then determined by X-ray crystallography to 1.8 and 1.9 Å resolution, respectively, to ascertain whether there were any differences in their interactions with the domain (Figure 1) that might be correlated with the observed difference in binding enthalpies of 2.0 kcal mol^{-1}. There are six complexes of **7** in the asymmetric unit, and these approximately isoenergetic complexes can be grouped into three conformational clusters that align with an average root-mean-square deviation (rmsd) of 0.5 Å for all backbone atoms with the primary difference being in the relative orientations of the BC loop. The backbone atoms in each of the six coexisting complexes of **7** align with those in the complex of **8** with similar rms deviations of 0.5−0.7 Å. As is evident upon examination of the superimposition in Figure 1a, the α-helices and β-sheets in the complexes align closely, but there are some significant variations in the flexible BC, CD, DE, and BG loops. The CD and DE loops are not involved in any contacts with the ligands, and the BG loop is involved in a single water-mediated protein−ligand contact in only two of the six coexisting complexes of **7**; hence, it is difficult to assess whether these structural dissimilarities affect relative binding energetics for **7** and **8**. The orientation of the BC loop, which is involved in numerous direct and water-mediated

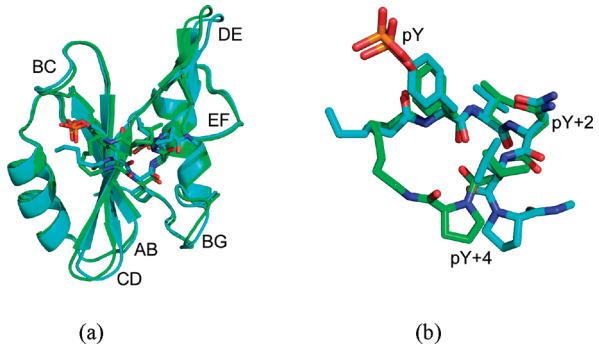

Figure 1. Images of **7** and **8** bound to the Grb2 SH2 domain following domain alignment. Oxygen, nitrogen, and phosphorus atoms are colored red, blue, and orange, respectively. Carbon atoms belonging to the complexes of **7** are colored green, while those belonging to the complex of **8** are colored cyan. Only the "a" complex of **7** is shown for clarity. (a) Image showing the complete domain (ribbons) and the bound ligands (sticks). (b) Image showing only the bound ligands (sticks).

contacts between the domain and **7** and **8**, varies noticeably in comparing the different complexes of **7** with **8**. However, these dissimilarities arise largely from differences in the six coexisting complexes of **7**, so correlating these differences with binding energetics of **7** and **8** is problematic. Structural alignments of the bound conformations of **7** and **8** reveal that those atoms belonging to the pYVN sequence align with an average rmsd of 0.3 Å (Figure 1b); they are thus virtually identical. There are, however, notable dissimilarities in the relative positions of other atoms in **7** and **8**, largely because the atoms in the N- and C-terminal moieties of **8** project away from each other.

Toward correlating energetics and structure, we inventoried the direct and single water-mediated polar contacts and the van der Waals contacts in the complexes of **7** and **8**. There is no difference in the number of direct polar contacts, but the number of water-mediated contacts in the six coexisting complexes of **7** and **8** varies by approximately ±1. However, these dissimilarities eventuate from differences in the number of ordered water molecules at the protein−ligand interface in the coexisting complexes of **7**, not from variations in the positions of atoms belonging to the ligand and the domain. There were also no discernible differences in van der Waals contacts between the domain and **7** and **8**. If

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one simply considers protein−ligand interactions, it is thus not possible to identify a sound structural basis for the observed differences in the binding enthalpies of **7** and **8**. There is, however, a notable difference in a hydrogen-bonding interaction in the bound structures of **7** and **8**. Namely, there is a hydrogen bond between the carbonyl oxygen atom of the pY residue and the backbone nitrogen atom of the pY+3 residue in **8** that is lacking in each of the six coexisting complexes of **7**; this intramolecular hydrogen bond is observed in the complexes of the Grb2 SH2 domain with a large number of pYVN-derived ligands.[3,6,7] The absence of this interaction suggests that **7** might bind in a higher energy conformation than **8**, a factor that is consistent with the observed enthalpic penalty of binding for **7** relative to **8**.

These results highlight our lack of understanding of energetics and structure in protein−ligand interactions, even in biological systems that are well-characterized. For example, comparing the binding enthalpies and entropies of the 23-membered macrocycles **3** and **7** with those of their respective linear controls **6** and **8** clearly demonstrates that ligand preorganization is not necessarily entropically favored as is widely posited. The nature and flexibility of the linker employed to create the macrocycle may play a role, even though this subunit does not interact with the protein, an interesting phenomenon that has been observed previously.[10] These studies also underscore the problems associated with correlating the number and/or type of protein−ligand contacts with specific contributions to binding enthalpies and entropies. Namely, the amino acid residues in **7** and **8** that interact with the domain have virtually identical structures, and they make the same direct contacts with the domain. The minor differences in the water-mediated contacts observed upon analyzing complexes of **7** and **8** arise from variations in the number of interfacial water molecules in the coexisting complexes of **7**; dissimilarities in these coexisting complexes are analogous to those found when comparing complexes of **7** and **8**.

There are, however, some structural features that may be correlated with binding affinities. For example, comparing the binding energetics of **4** and **5** reveals that introducing a valine residue at the pY+3 position had a favorable impact on binding free energy as predicted and that this enhanced affinity is entropy driven is consistent with the classical hydrophobic effect. These structural studies also suggest that the reduced affinities of the 20-membered macrocycles might be attributed in part to their failure to form direct contacts with the domain that are highly conserved in other complexes. Finally, the enthalpic penalty associated with the binding of the macrocycle **7** relative to its linear control **8** might be partially attributed to the absence of an intramolecular hydrogen bond that could contribute to the stability of the bound conformation of **7**.

SUPPORTING INFORMATION AVAILABLE Experimental procedures, spectral data, and copies of ^{1}H and ^{13}C NMR spectra for all new compounds; plots of ΔH° vs T for **3** and **6**−**8**; complete diffraction data and refinement statistics, density difference maps and omit maps, protein−ligand polar contact diagrams, and tables listing the number of direct and water-mediated protein−ligand contacts in the complexes of **7** and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes: Coordinates and structure factors for the Grb2 SH2 domain in complex with **2**, **7**, and **8** have been deposited in the RCSB Protein Data Bank as entries 3N7Y, 3N84, and 3N8M, respectively.

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