ORGANIC LETTERS

2004
Vol. 6, No. 20
3429−3432

Thermodynamic Analysis of a Hydrophobic Binding Site: Probing the PDZ Domain with Nonproteinogenic Peptide Ligands

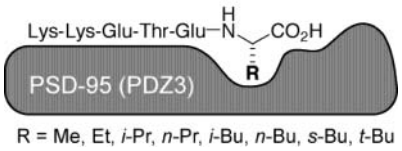
Dorina Saro, Edvin Klosi, Azrael Paredes, and Mark R. Spaller*

Department of Chemistry, Wayne State University, Detroit, Michigan 48202

mspaller@chem.wayne.edu

Received May 4, 2004

ABSTRACT



Isothermal titration calorimetry (ITC) is used to study the thermodynamic consequences of systematically modifying the hydrophobic character of a single residue in a series of protein-binding ligands. By substituting standard and nonproteinogenic aliphatic amino acids for the C-terminal valine of the hexapeptide KKETEV, binding to the third PDZ domain (PDZ3) of the PSD-95 protein is characterized by distinct changes in the Gibbs free energy (ΔG), enthalpy (ΔH), and entropy ($T\Delta S$) parameters. One notable observation is that peptide binding affinity can be improved with a nonstandard residue.

What is the contribution to binding of a single functional group in a protein−ligand interaction? The simplicity of this question belies the complexity involved in both unambiguously and quantitatively correlating molecular structure with binding energetics. In this paper, we investigate the most fundamental of the organic functional groups—the hydrocarbon unit. While not endowed with the electronic character that allows the more polar functional groups to participate in hydrogen-bonding or electrostatic-based interactions, methylene (−CH_2−) and methyl (−CH_3) groups often contribute profoundly to the binding thermodynamics of protein−ligand interactions through the hydrophobic effect and van der Waals forces.[1]

To study the behavior of these units in the context of a relevant model, we sought not only a protein−ligand system that possessed a well-defined aliphatic binding determinant but also one in which the nonpolar interaction contributes prominently to an association of cellular importance and for which the design of synthetic binding ligands is an important goal. These multiple requirements are fulfilled by the third PDZ domain (PDZ3) from the *postsynaptic density 95 kDa* (PSD-95) protein.

PSD-95 resides at the crossroads of numerous protein−protein interactions that occur at the neuronal synapse.[2] With its selective clustering ability, PSD-95 forms transient assemblies that can involve cell membrane-bound receptors, which include interactions that are mediated by the three nonidentical PDZ domains found in the protein. In general, PDZ domains endow their host polypeptide with the ability to mediate specific protein−protein interactions.[3] These associations usually occur at the C-terminus of the partner protein, which can often be mimicked using short peptides that possess the same C-terminal sequence.

Like other class I PDZ domains, the third PDZ domain (PDZ3) of PSD-95 recognizes a "*X*-Ser/Thr-*X*-Val-CO_2H" tetrapeptide motif (where *X* represents variable residues and Val is at the C-terminus). When viewing the structure of

(1) Davis, A. M.; Teague, S. J. *Angew. Chem., Int. Ed.* **1999**, *38*, 736−749.

(2) Irie, M.; Hata, Y.; Takeuchi, M.; Ichtchenko, K.; Toyoda, A.; Hirao, K.; Takai, Y.; Rosahl, T. W.; Sudhof, T. C. *Science* **1997**, *277*, 1511−1515.

(3) (a) Sheng, M.; Sala, C. *Annu. Rev. Neurosci.* **2001**, *24*, 1−29. (b) Harris, B. Z.; Lim, W. A. *J. Cell Sci.* **2001**, *114*, 3219−3231.

10.1021/ol049181q CCC: $27.50
Published on Web 08/28/2004

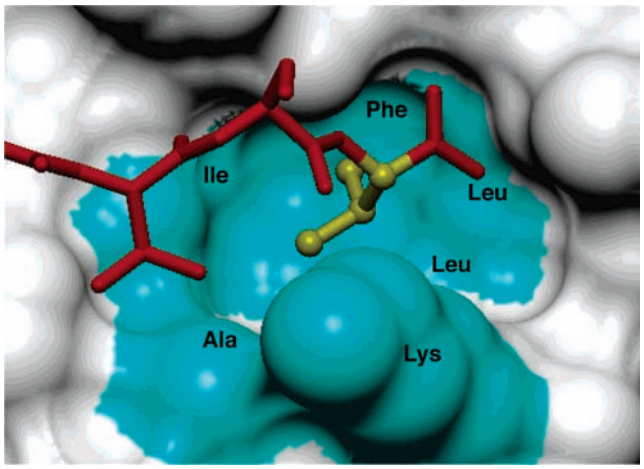


Figure 1. Binding pocket of PDZ3 for the C-terminal Val side chain (yellow) of a bound peptide (red). Nonpolar PDZ3 residues (blue) are labeled in which at least one heavy atom resides within 5 Å of the Val side chain. Although Lys contains a basic amine, several of the side chain methylene units are in proximity to Val. Modeling was performed with the PDZ3−peptide complex (PDB code: 1BE9) using Insight II.

PDZ3 in complex with the peptide Lys-Gln-Thr-Ser-Val,[4] the valine isopropyl side chain is seen jutting into a nonpolar pocket in the protein (Figure 1). This conserved interaction bolstered the early hypothesis that the terminal ligand residue is important for imparting selectivity in binding to PDZ3 and, by extension, to other class I PDZ domains.[4]

Our approach was to select a reference PDZ3-binding peptide with a C-terminal valine, which would then be substituted with both standard and nonproteinogenic[5] residues that possess increasing aliphatic character. The aim is to observe the change in binding behavior when the side chain is modified in one of two ways: through introducing additional methylene or methyl groups and by shifting the position of existing methyl groups. In this manner, incremental structural modifications can be made that are not accessible if only the standard 20 amino acids are employed. The use of peptides with nonproteinogenic residues to examine the thermodynamic parameters of protein−ligand interactions is surprisingly underutilized.[6] This study is the first to report the changes in enthalpy and entropy that occur when systematic modifications are made to a specific nonpolar binding determinant within a protein−peptide interaction. In a similar vein, we have recently described the thermodynamic profile for a series of novel macrocylic ligands for the PDZ domain; in that investigation, the ring

(4) Doyle, D. A.; Lee, A.; Lewis, J.; Kim, E.; Sheng, M.; MacKinnon, R. *Cell* **1996**, *85*, 1067−1076.

(5) Abbreviations for the nonproteinogenic amino acids: Abu, 2-aminobutanoic acid; Aib, α-aminoisobutyric acid; Ahx, 2-aminohexanoic acid (Nle, norleucine); Ape, 2-aminopentanoic acid (Nva, norvaline); Tbg, *tert*-butyl glycine (Tle, *tert*-leucine, or 2-amino-3,3-dimethylbutyric acid).

(6) (a) Bigler, T. L.; Lu, W.; Park, S. J.; Tashiro, M.; Wieczorek, M.; Wynn, R.; Laskowski, M., Jr. *Protein Sci.* **1993**, *2*, 786−799. (b) Huang, K.; Lu, W.; Anderson, S.; Laskowski, M., Jr.; James, M. N. *Protein Sci.* **1995**, *4*, 1985−1997.

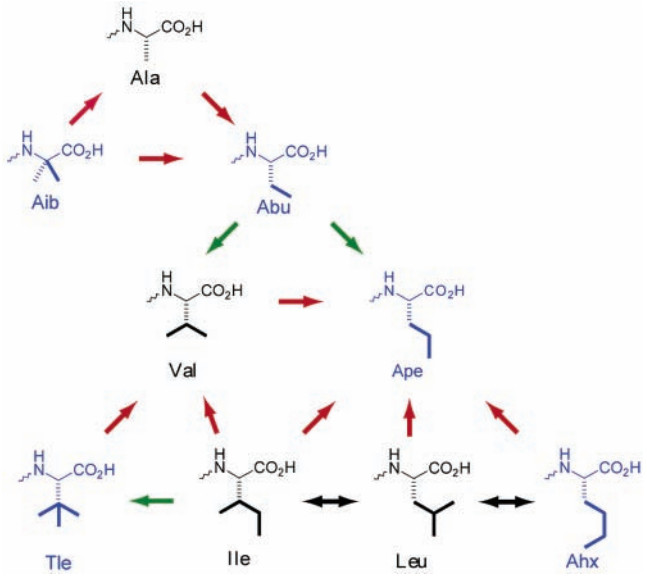


Figure 2. Linear and acyclic branched standard (black) and nonproteinogenic (blue) amino acids incorporated into the C-terminal position (*X*) of the KKETE*X* peptide series. Residues immediately horizontal to one another have an isomeric ("CH_3-shift") relationship; residues separated vertically differ by single-carbon unit additions ("CH_3− addition", or more accurately, a "CH_2− insertion" into an existing C−H bond). Arrow direction indicates a transition that improves ΔG primarily through ΔH (red) or $T\Delta S$ (green) changes; black arrows reflect equal contributions within experimental error.

size was systematically expanded and contracted, primarily through the addition of methylene equivalents.[7]

We selected as a reference ligand the hexapeptide KKETEV, designed on the basis of preferred binding sequences for class I domains, which exhibits good affinity for PDZ3 (K_d ~2 μM). Analogues were then synthesized whereby the terminal Val was replaced with residues bearing linear and branched aliphatic side chains, such that each modification represents an *addition* or *isomerization* of a hydrocarbon unit. (Figure 2).

Collectively, the residues possess the common carbogenic substituents *methyl*, α,α-*dimethyl*, *ethyl*, *isopropyl*, *n-propyl*, *isobutyl*, *n-butyl*, *sec-butyl*, *and tert-butyl*. Each peptide of this KKETE*X* series (where *X* is the variable residue) was individually synthesized; the binding of the purified peptides to recombinantly prepared PDZ3 domain protein was then evaluated by isothermal titration calorimetry (ITC). An ideal method to evaluate protein−ligand interactions in solution, ITC provides in a single experiment the full panel of thermodynamic parameters, i.e., the Gibbs free energy change (ΔG) and the changes in enthalpy (ΔH) and entropy (ΔS).[8]

Figure 3 and Table 1 display the data from the ITC experiments using the nine KKETE*X* peptides. The affinity

(7) Li, T.; Saro, D.; Spaller, M. R. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1385−1388.

(8) (a) Leavitt, S.; Freire, E. *Curr. Opin. Struct. Biol.* **2001**, *11*, 560−566. (b) Ward, W. H.; Holdgate, G. A. *Prog. Med. Chem.* **2001**, *38*, 309−376.

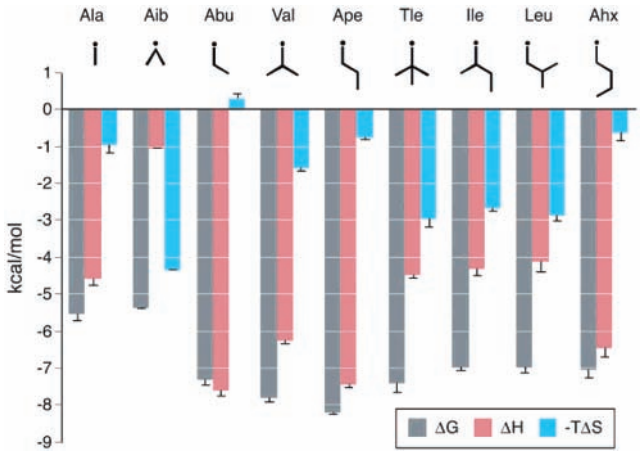


Figure 3. Bar chart with ΔG, ΔH, and $-T\Delta S$ values for each member of the KKETE*X* series (data from Table 1). $T\Delta S$ is graphed as the negative value.

of the reference ligand KKETEV exceeds that of the ligands with the other standard residues (Ala, Leu, and Ile) at the terminal position. This is consistent with the general frequency with which these residues are encountered in protein binding partners of class I PDZ domains. Yet an improvement in ΔG is possible; the nonproteinogenic straight-chain isomer of valine, Ape, yields a 2-fold binding enhancement with a K_d of 1 μM. The weakest hexapeptide, containing a terminal Ala, is separated from the strongest binding Ape derivative by close to 2 orders of magnitude, and yet the difference is simply a pair of carbon units at the side chain—methyl versus *n*-propyl. This illustrates well how seemingly modest changes in the carbogenic makeup of a single side chain can have a substantial impact upon the protein-binding properties of a peptide.

Looking beyond the affinity as represented by ΔG, the underlying ΔH and ΔS values can be interpreted to provide insight into the nature of the binding interaction. These parameters can be particularly informative when comparing the differences between two or more ligands (i.e., $\Delta\Delta H$ and $T\Delta\Delta S$). To clearly depict this for each possible ligand transition, a matrix showing all pairwise differences was constructed (Figure 4).

The weakest binding ligands are those containing a C-terminal Ala and Aib, which exhibit comparable affinities. What is striking about this pair is that the enthalpy and

Table 1. Thermodynamic Binding Parameters for the Calorimetric Titration of KKETE*X* into PDZ3 from PSD-95[a]

X (side chain)	K_d[b] (μM)	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)
Ala	91.4	−5.51	−4.56	0.95
(Me)	(±1.7)	(±0.01)	(±0.22)	(±0.21)
Aib	116	−5.37	−1.03	4.34
(Me, Me)	(±1)	(±0.01)	(±0.02)	(±0.01)
Abu	4.47	−7.31	−7.61	−0.30
(Et)	(±0.88)	(±0.11)	(±0.15)	(±0.14)
Val	1.93	−7.80	−6.23	1.57
(*i*-Pr)	(±0.04)	(±0.01)	(±0.12)	(±0.11)
Ape	1.01	−8.18	−7.42	0.76
(*n*-Pr)	(±0.07)	(±0.04)	(±0.11)	(±0.06)
Tle	3. 88	−7.41	−4.47	2.94
(*t*-Bu)	(±0.11)	(±0.17)	(±0.11)	(±0.25)
Ile	7.70	−6.98	−4.31	2.67
(*s*-Bu)	(±1.2)	(±0.09)	(±0.19)	(±0.10)
Leu	7.88	−6.97	−4.12	2.85
(*i*-Bu)	(±1.3)	(±0.10)	(±0.28)	(±0.18)
Ahx	6.98	−7.03	−6.43	0.61
(*n*-Bu)	(±0.52)	(±0.04)	(±0.27)	(±0.25)

[a] Values are the mean of at least two independent experiments (error shown below reflects the range). Binding stoichiometry values (n) ranged from 0.9 to 1.1. No significant proton coupling was observed with KKETEV in experiments with different buffers (data not shown), so it is assumed that $\Delta H \approx \Delta H_{obs}$.

X side chain	Ala	Aib	Abu	Val	Ape	Tle	Ile	Leu	Ahx
Ala	—	-0.1	1.8	2.3	2.7	1.9	1.5	1.5	1.5
Aib	3.5 *3.4*	—	1.9	2.4	2.8	2.0	1.6	1.6	1.7
Abu	-3.0 *-1.3*	-6.6 *-4.6*	—	0.5	0.9	0.1	-0.3	-0.3	-0.3
Val	-1.7 *0.6*	-5.2 *-2.8*	1.4 *1.9*	—	0.4	-0.4	-0.8	-0.8	-0.8
Ape	-2.9 *-0.2*	-6.4 *-3.6*	0.2 *1.1*	-1.2 *-0.8*	—	-0.8	-1.2	-1.2	-1.1
Tle	0.1 *2.0*	-3.4 *-1.4*	3.1 *3.2*	1.8 *1.4*	3.0 *2.2*	—	-0.4	-0.4	-0.4
Ile	0.3 *1.7*	-3.3 *-1.7*	3.3 *3.0*	1.9 *1.1*	3.1 *1.9*	0.2 *-0.3*	—	0.0	0.0
Leu	0.4 *1.9*	-3.1 *-1.5*	3.5 *3.2*	2.1 *1.3*	3.3 *2.1*	0.4 *-0.1*	0.2 *0.2*	—	0.1
Ahx	-1.9 *-0.3*	-5.4 *-3.7*	1.2 *0.9*	-0.2 *-1.0*	1.0 *-0.2*	-2.0 *-2.3*	-2.1 *-2.1*	-2.3 *-2.2*	—

Figure 4. Pairwise differences in thermodynamic values for the KKETE*X* peptides. The axes display the side-chain structure for the variable *X* residue. $\Delta\Delta G$ (top right portion), $\Delta\Delta H$ (bottom left portion, upper value), and $T\Delta\Delta S$ (bottom left portion, lower value in *italics*) values are shown. All signs pertain to the transitions from the top row (horizontal) ligands to those in the left column (vertical). Shaded cells denote "C-isomerizations" (lighter shade) and "C-additions" (darker shade). Units are in kcal/mol.

entropy values switch quite dramatically: the Ala compound is driven by a favorable ΔH of -4.6 kcal/mol, whereas the major contribution to ΔG of the Aib analogue is entropic, with $T\Delta S$ of 4.3 kcal/mol. As the only α,α-dialkylated residue in this series, Aib might be effecting this enthalpy−entropy compensation[9] behavior through a Thorpe−Ingold (*gem*-dimethyl) effect in which the peptide backbone experiences a biased conformation.[10]

Addition of carbon units to the side chain is expected to influence affinity dramatically, since there is presumably an ideal volume for optimal packing of a residue side chain.[11] The largest jump in affinity occurs when Ala is changed to Abu, which is close to the value measured for the native Val. This gain is due to a $\Delta\Delta$H on the order of -3 kcal/mol, and the change in entropy is actually disfavored. The added methyl might not necessarily be contributing to the driving force via a classical hydrophobic effect (removal of bound water). If it is, the ΔS value may be masked by the conformational cost of restricting the ethyl group within the binding site. In general, interpretations of entropic change are often multifold and offsetting; e.g., pitting the favorable effect of desolvation against the penalty incurred by restricted bond rotations.

The effect of removing a single β-methyl from Val to form the Abu derivative results in a modest 2-fold drop in K_d. This ligand exhibits the *least favorable* change in entropy, with a $T\Delta S$ drop of 1.9 kcal/mol. One possible interpretation is that the more flexible Abu side chain ("ethyl") requires restriction to adopt a comfortable binding posture, whereas the Val side chain ("isopropyl") is partially restricted due to β-substitution.

In all but two cases, adding a single carbon to an existing side chain results in an improvement in binding entropy ($+T\Delta\Delta S$). A global interpretation for this is the favorable desolvation of ordered waters by the larger hydrophobic side chain upon binding. Alternatively, in the subset of cases in which addition of the carbon results in an added site of branching, the explanation could also involve the increased rotational restriction of the side chain and, thus, a lowered entropic penalty upon binding.

In considering the effect of *shifting* carbon units, aside from the atypical case of Aib, the isomerization of the side chain does not have a large effect on ΔG. The branched-to-linear Val → Ape transition, which yields the highest affinity ligand of the series, is marked by a decrease in ΔH and a near-compensating drop in $T\Delta S$. An entropic improvement is likewise observed when other equal-carbon residue ligand pairs isomerize from a more to a less "congested" branching isoform (i.e., Aib → Abu, Val → Ape, Tle → Ile, Ile → Ahx, Leu → Ahx); $T\Delta\Delta S$ values range from slightly (-0.3) to very (-4.6) unfavorable. In fact, a survey of all the ligands in Table 1 shows that the most positive $T\Delta S$ values belong to the ligands with branched residues. This suggests that the more branched side chains impart a preorganization that favors the binding mode, and that increasing the rotational freedom by reducing branching is entropically costly. Conversely, when isomerizing from a branched to a linear residue (e.g., Val → Ape, Tle/Leu/Leu → Ahx), the resulting ligands improve in ΔH. This may be attributable to increased flexibility that can maximize interactions in the binding cavity.

Whether these observations based on a limited compound set will point to a general "thermodynamic design" principle for peptide ligands—for example, that increasing the degree of side chain branching for a residue with a fixed number of side chain carbons will lead to entropic improvements in binding—will have to await further studies, since this type of analysis is largely absent from the literature.

This brief summary on selected residues and trends provides an overview on how this approach provides quantitative insight into protein−peptide molecular recognition and supports the prospect of "fine-tuning" such interactions with nonproteinogenic residues. With respect to binding to PDZ3, and more broadly to other class I PDZ domains, the data underscore the significance of incremental changes in the carbogenic side-chain structure at a single binding pocket. In addition, the development of more selective, higher affinity peptide ligands for PDZ domains may be fostered through the use of nonstandard residues. Further, these results may contribute to decisions on combinatorial library design, such as in removal of isoenergetic residues, or, more intriguingly, enriching starting material input with residues that may lead to ligands with a particular enthalpic or entropic bias.

Acknowledgment. Funding for this research was provided by the National Institutes of Health (GM63021).

Supporting Information Available: Experimental procedures for PDZ3 expression, purification, and ITC binding analysis; characterization of nonproteinogenic peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

OL049181Q

(9) Sharp, K. *Protein Sci.* **2001**, *10*, 661−667.
(10) Toniolo, C.; Crisma, M.; Formaggio, F.; Peggion, C. *Biopolymers* **2001**, *60*, 396−419.
(11) Harpaz, Y.; Gerstein, M.; Chothia, C. *Structure* **1994**, *2*, 641−649.