Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 1385–1388

# Thermodynamic profiling of conformationally constrained cyclic ligands for the PDZ domain

Tao Li, Dorina Saro and Mark R. Spaller*

*Department of Chemistry, Wayne State University, Detroit, MI 48202, USA*

Received 7 July 2003; accepted 8 September 2003

**Abstract**—Inspired by structure-based design and tailored for combinatorial preparation, a series of novel cyclic peptides has been developed to yield binding ligands for the third PDZ domain (PDZ3) of PSD-95. These side chain–side chain bridged peptides permit the systematic expansion or contraction of ring size, which is intended to maximize the conformational diversity of the ensemble. Isothermal titration calorimetry (ITC) was used to measure the dissociation constants ($K_d$) and associated thermodynamic binding parameters.


## 1. Introduction

PDZ domains mediate a variety of protein–protein interactions in eukaryotic cells, most notably those found within signaling complexes at the mammalian plasma membrane.[1] As *adaptor* or *scaffold* proteins, these domains share homologous sequences that adopt autonomous tertiary structure within larger polypeptides. This arrangement allows the host protein to recognize one or more endogenous ligands, modulating the activity of numerous cellular processes.[2] PDZ domains have been implicated in a variety of associations involving proteins of medicinal interest; these include the oncogenic human papillomavirus *E6 proteins*,[3] metastatic breast cancer *syntenin*,[4] prostate cancer *AIPC*,[5] and Parkinson's disease *parkin*.[6]

From a biophysical perspective, PDZ domains exhibit a unique molecular recognition behavior: an almost singular preference for binding the C-terminal region of their protein partners, coupled with an ability to discriminate amongst them based on the identity and position of only a handful of residues. In an effort to better understand the underpinnings of this binding character, and in turn to develop selective ligands to serve as molecular probes for future studies, we report here the first thermodynamic investigation of a series of conformationally constrained, small cyclic peptides that target a PDZ domain.

* Corresponding author. Tel.: +1-313-577-3336; fax: +1-313-577-8822; e-mail: mspaller@chem.wayne.edu

## 2. Ligand design

The third PDZ domain (PDZ3) of the *postsynaptic density-95 kDa* protein (PSD-95, also termed SAP90) was selected as a model system for ligand design. PSD-95 occupies a prominent role in protein clustering at neuronal membranes, and in a demonstration of therapeutic relevance, disrupting the PDZ-mediated association of PSD-95 with the NMDA receptor was shown to reduce the severity of ischemic brain damage associated with stroke in animal models.[7]

PDZ3 has been structurally characterized bound to a nine-residue peptide,[8] which corresponds to the C-terminal sequence of the CRIPT protein.[9] During the course of molecular modeling studies with this complex, we hypothesized that by maintaining the preferred identities of the C-terminal ($P_0$) and antepenultimate ($P_{-2}$) residues—those engaged in pronounced contacts with the protein—and by tethering the more solvent-accessible ($P_{-1}$ and $P_{-3}$) residue side chains, the end result would be a conformationally constrained macrocyclic ligand (Fig. 1).

Although many options exist for short-range cyclization of linear peptides,[10] we reasoned that the commonly employed *direct* linkage of side chains (such as the side chain-side chain Lys/Glu amide or Cys/Cys disulfide bonds) might impart an unfavorable distortion of the peptide backbone away from an optimal binding geometry. Instead, a *bridging* approach was pursued, in which the $P_{-1}$ and $P_{-3}$ side chains are covalently

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.09.103

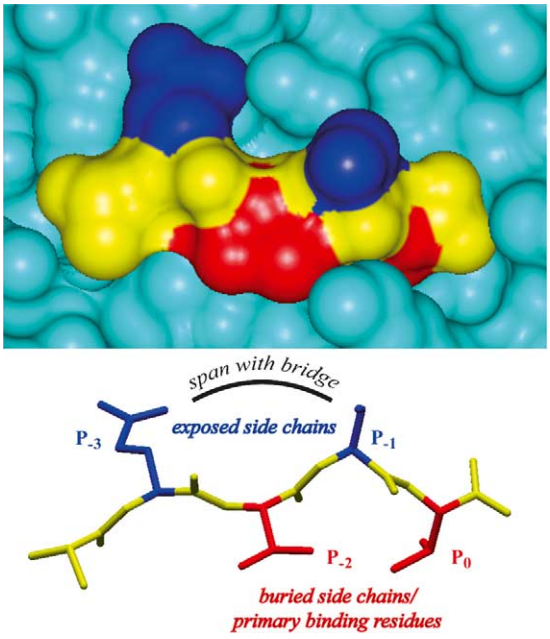



**Figure 1.** Design concept for bridged cyclic ligands based on the X-ray crystallographic structure of PDZ3-bound Gln-Thr-Ser-Val.

spanned by a segment of desired length. To reduce the concept to practice, a ligand was designed based on the C-terminal six residues of CRIPT, Tyr-Lys-Gln-Thr-Ser-Val. The $P_{-3}$ and $P_{-1}$ residues were altered, respectively, from Gln to an amine-bearing *X* and Ser to a carboxylate-bearing *Y*, where *X* and *Y* are standard or nonstandard amino acids. These side chains each form an amide bond with an introduced bridge residue, an α-, β- or γ-amino acid (Fig. 2).

This flexible design allows for attenuation of ring size through substitution at three sites: residues $P_{-1}$ and $P_{-3}$, and the bridge. By expanding or contracting the macrocycle, it is postulated that each compound in an enumerated library would adopt a distinct conformation (or nonidentical ensemble of conformations). The key binding determinants for PDZ3 (Val at $P_0$ and Thr at $P_{-2}$) are kept in place. Overall, this should promote more thorough conformational coverage of these ligands by spatially repositioning, to varied degrees, the $P_0$ and $P_{-2}$ side chains. Thus, while each *individual* ligand would possess a certain measure of conformational constraint, *collectively* the library exhibits conformational diversity.

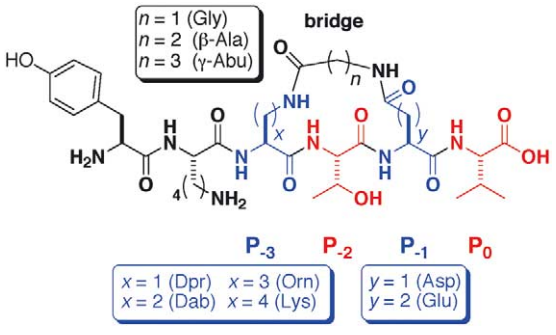


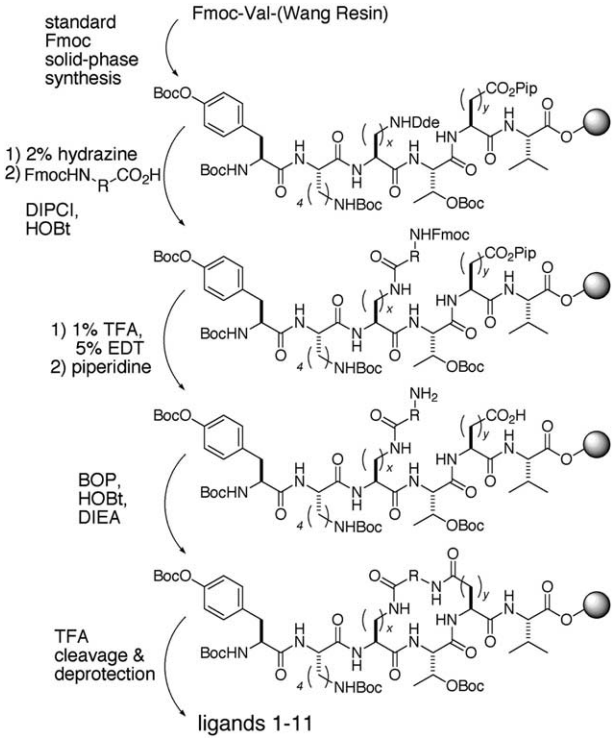

**Figure 2.** Cyclic ligand design depicting the three substitutable positions ($P_{-1}$, $P_{-3}$, the bridge, and the amino acids used), and two fixed binding residues ($P_0$, $P_{-2}$) important for PDZ3 binding.

## 3. Results and discussion

A solid-phase synthetic route was developed that incorporated the described design features (Scheme 1). While the method relies largely upon standard Fmoc-based peptide chemistry, the manner of assembly requires judicious placement of key residues with suitably orthogonal protecting groups (Dde[11] and Pip[12]). In addition, a survey was conducted of several coupling reagents for effecting the on-bead macrocyclization, in order to identify conditions such that each ring size product would be generated in reasonable quantity.

Eleven of the possible 24 cyclic compounds depicted in Figure 2 were individually prepared, and their binding to PDZ3 evaluated using isothermal titration calorimetry (ITC) (Fig. 3 and Table 1). ITC provides a full thermodynamic profile [changes in Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (ΔS)] for each binding event, including the stoichiometry.[13] Further benefits of using ITC are that it is a true solution method with no surface immobilization and that no added tags or labels are required for signal detection. Although a few recent reports using native and modified linear peptides have determined affinity values,[14] there are no accounts that have focused on the underlying thermodynamics of PDZ domain–ligand interactions.

In the absence of explicit structural characterization of the protein–macrocycle complexes, assigning specific molecular interactions to measured ΔH and TΔS values




**Scheme 1.** Solid-phase synthetic scheme for cyclic ligands.

**Table 1.** Thermodynamic binding parameters for the binding of cyclic and linear control peptide ligands (Fig. 3) to PDZ3 from PSD-95[a]

| Ligand | $x$ ($P_{-3}$ residue) | $y$ ($P_{-1}$ residue) | R (Bridge residue) | Z | ring size | $K_d$ (μM) | ΔG (kcal/mol) | ΔH (kcal/mol) | TΔS (kcal/mol) |
|---|---|---|---|---|---|---|---|---|---|
| 1[b] | 1 (Dpr) | 1 (Asp) | $-CH_2$-(Gly) | –NH– | 14 | — | — | — | — |
| 2 | 2 (Dab) | 1 (Asp) | $-CH_2$-(Gly) | –NH– | 15 | 61.2 (±7.0) | −5.74 (±0.06) | −2.06 (±0.32) | 3.69 (±0.38) |
| 3[b] | 3 (Orn) | 1 (Asp) | $-CH_2$-(Gly) | –NH– | 16 | — | — | — | — |
| 4 | 4 (Lys) | 1 (Asp) | $-CH_2$-(Gly) | –NH– | 17 | 65.1 (±1.7) | −5.71 (±0.02) | −1.22 (±0.14) | 4.46 (±0.15) |
| 5 | 4 (Lys) | 2 (Glu) | $-CH_2$-(Gly) | –NH– | 18 | 18.5 (±3.5) | −6.45 (±0.10) | −2.47 (±0.09) | 4.00 (±0.01) |
| 6 | 1 (Dpr) | 1 (Asp) | $-CH_2CH_2$-(β-Ala) | –NH– | 15 | 29.6 (±2.9) | −6.17 (±0.05) | −2.95 (±0.08) | 3.23 (±0.02) |
| 7 | 4 (Lys) | 1 (Asp) | $-CH_2CH_2$-(β-Ala) | –NH– | 18 | 34.5 (±3.1) | −6.08 (±0.01) | −2.45 (±0.01) | 3.64 (±0.06) |
| 8 | 4 (Lys) | 2 (Glu) | $-CH_2CH_2$-(β-Ala) | –NH– | 19 | 4.47 (±0.26) | −7.29 (±0.03) | −2.29 (±0.01) | 5.01 (±0.03) |
| 9 | 4 (Lys) | 2 (Glu) | $-CH_2CH_2CH_2$-(γ-Abu) | –NH– | 20 | 9.16 (±0.61) | −6.87 (±0.04) | −2.75 (±0.12) | 4.13 (±0.16) |
| 10 | 4 (Lys) | 2 (Glu) | –m-$C_6H_4$-(m-Abz) | –NH– | 20[c] | 19.7 (±3.4) | −6.41 (±0.09) | −1.42 (±0.12) | 5.01 (±0.02) |
| 11 | 4 (Lys) | 2 (Glu) | –CH(*S*-Bn)$CH_2$-(β-homoPhe) | –NH– | 19 | 6.15 (±0.43) | −7.10 (±0.04) | −3.39 (±0.33) | 3.72 (±0.03) |
| 12 | 4 (Lys) | 2 (Glu) | $-CH_2CH_2NH_2$ | HO– | — | 5.54 (±0.18) | −7.17 (±0.02) | −4.77 (±0.35) | 2.40 (±0.30) |
| 13 | 4 (Lys) | 1 (Asp) | $-CH_2CH_2NH_2$ | HO– | — | 44.1 (±5.7) | −5.94 (±0.07) | −1.38 (±0.07) | 4.57 (±0.15) |
| 14 | 4 (Lys) | 1 (Asp) | $-CH_2NH_2$ | HO– | — | 53.3 (±9.7) | −5.83 (±0.10) | −1.62 (±0.13) | 4.22 (±0.02) |
| 15 | 4 (Lys) | 2 (Glu) | $-CH_3$ | $H_2N$– | — | 5.03 (±0.18) | −7.22 (±0.02) | −3.08 (±0.09) | 4.15 (±0.07) |

[a] Values are the average of two independent experiments performed at 25 °C. Stoichiometry ($n$) values ranged from 0.85 to 1.10.
[b] Insufficient heat was generated in these titrations to reliably determine binding parameters.
[c] Defined based on the path of fewest carbons through the aromatic ring.

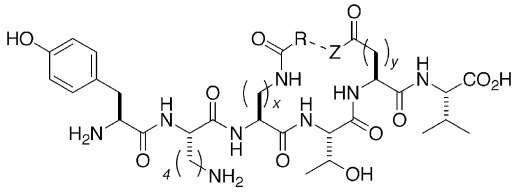


**Figure 3.** Template for cyclic ligands described in Table 1. R–Z represents the bridge covalent bond in the cyclic compounds, and a 'nonexistant bond' for the linear control peptides.

is a speculative undertaking, and all such interpretations remain provisional.[13b] In addition, the sampling of compounds tested is only a subset of larger possible libraries, so broader trends and patterns may remain undetected. These caveats aside, the thermodynamic data obtained provides preliminary insight into the nature of macrocycle binding to PDZ3 (Table 1).

Dissociation constants for the ligands varied over an order of magnitude, from 4.5 to 65 μM. The range of ΔH and TΔS values — which were both favorable to binding for all cases — extended over ca. 2.2 and 1.8 kcal/mol, respectively. Slight modification of the ring size has a significant effect not only on $K_d$, but also in the partitioning of ΔH and TΔS values. Comparing the transitions **4**→**5** and **4**→**7** (where the ring increases from 17 to 18 atoms by inserting a methylene group in different locations), the larger ring ligands (**5** and **7**) differ by $\Delta\Delta G \cong 0.4$ kcal/mol. When modifying the bridge length of the highest affinity ligand **8**, either removing (**5**) or adding (**9**) a -$CH_2$- unit leads to diminished affinity. Even when the ring size and amide bond inventory are kept constant, a two-fold change in $K_d$ is observed (**2** and **6**, **5** and **7**).

Functionalization of the bridge to elicit additional favorable interactions remote from the recognized binding cavity is attempted with benzyl-substituted **11** and the *m*-aminobenzoic acid derivative **10**. Although the affinity is comparable to **8**, **11** displays the most favorable change in ΔH of the cyclic compounds. This strategy of modifying the distal region of PDZ-binding ligands may ultimately allow for more selectivity when targeting one particular PDZ domain in the presence of numerous others of the same class.

Several linear controls (**12**–**15**) were prepared to gauge the value of cyclization itself, particularly since the bridging fragment may introduce additional interactions with the protein. Several compound pairs (**4**→**14**, **7**→**13**, **8**→**12**) represent a formal hydrolysis (at the $P_{-1}$ side chain amide) that converts a cyclic to linear ligand. For each linear-cyclic pair, the affinity values are comparable, well under an order of magnitude difference. There is a large, favorable increase in TΔΔS (2.6 kcal/mol) moving from linear **12** to cyclic **8**. While this might be ascribable to a decreased entropic penalty of binding (through rotor restriction), it is also possible that the binding of the cyclic ligand results in more favorable solvent reorganization.

This observation, however, is not replicated in pairs **4**/**14** and **7**/**13**. It should be noted that the controls are unavoidably imperfect, since breaking the ring in a formal hydrolysis creates ionizable amine and carboxylate groups; these may participate in dramatically different interactions with the protein. Linear **15** represents a formal '$H_2$ reduction' of the bridge N-$C_\alpha$ of cyclic **5**, and actually improves affinity slightly, primarily through an enthalpic gain.

In summary, we have demonstrated that small cyclic peptides can serve as ligands for the PDZ domain. The modular composition of the macrocycle supports a combinatorial approach towards preparation, leading to collections of compounds designed to exhibit diversity in terms of both function and conformation. Calorimetric measurements show that apparently minor changes in ring size can notably influence the thermodynamic binding parameters. These data will complement and assist future studies that include PDZ-ligand structural characterization, selectivity studies with other PDZ domains, and the development of next-generation ligands designed to elucidate the underlying molecular forces involved in PDZ domain-binding interactions.

## 4. Solid-phase synthesis of ligands

The fully linear, orthogonally protected peptides were prepared using standard manual Fmoc peptide synthesis protocols (DIPCI, HOBt) starting with preloaded Val (Wang resin), except that the final residue added (Tyr) bore an N-terminal Boc protecting group. Hydrazine (2% in DMF) was used to remove the Dde group, which was confirmed by the Kaiser test. Attachment of the bridge (using the desired Fmoc-protected amino acid) to the exposed side chain amine was accomplished using standard Fmoc protocols. Pip deprotection (1% TFA/5% EDT in DCM, 4×15 min washes)[15] was followed by a final, thorough DMF wash. The bridge residue Fmoc group was removed with piperidine and the resin washed with DMF. The resin beads often aggregated at this point, and the resin was washed three times with DIPEA in DCM (1:9 v/v, 3×3 min) followed by a DMF wash. DMSO/*N*-methylpyrrolidone (NMP) (1:4 v/v) was added to the reaction vessel followed by gentle shaking until the aggregated resin became evenly suspended. BOP (3 equiv.), HOBt (3 equiv.) and DIPEA (6 equiv.) were added. The reaction was allowed to proceed for 2 h (if the Kaiser test suggested incomplete coupling, the resin was washed (DMF) and treated once more with the coupling conditions). The peptide was cleaved from resin (TFA/EDT, 95:5 v/v), precipitated and washed with diethyl ether, and the precipitate dissolved in water. Lyophilization yielded a white, loose powder that was purified by reverse-phase HPLC, and the isolated compounds confirmed by electrospray ionization-MS.[16]

## 5. ITC experiments

PDZ3 was obtained by selective trypsin cleavage at a thrombin recognition site of bacterially-expressed GST-PDZ3 fusion protein, then purified by ion-exchange chromatography. Experiments were performed with a VP-ITC titration microcalorimeter (Microcal, Inc.). PDZ3 samples were thoroughly dialyzed, and the ligands dissolved in the final dialysis buffer. All experiments were conducted at 25 °C in 20 mM MES (pH 6.0) with 10 mM NaCl. The pH values for PDZ3 and ligand solutions were matched within 0.02 pH units, and solutions were degassed. For a typical titration, the ligand (290 μL, 1.2–2.0 mM) was injected into PDZ3 (ca. 1.4 mL, 100–140 μM). Heats of dilution were measured in blank titrations (peptides into buffer) and subtracted from the binding heat values. ORIGIN software (version 5.0, Microcal) was used for data collection and analysis.

## Acknowledgements

We thank R. MacKinnon (Rockefeller Univ.) for the GST-PDZ3 expression vector, and L. Hryhorczuk (WSU) for mass spectroscopy services. This work was supported by the National Institutes of Health (GM63021).

## References and notes

1. (a) Sheng, M.; Sala, C. *Ann. Rev. Neurosci.* **2001**, *24*, 1. (b) Harris, B. Z.; Lim, W. A. *J. Cell. Sci.* **2001**, *114*, 3219.
2. Pawson, T.; Scott, J. D. *Science* **1997**, *278*, 2075.
3. (a) Kiyono, T.; Hiraiwa, A.; Fujita, M.; Hayashi, Y.; Akiyama, T.; Ishibashi, M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11612. (b) Nguyen, M. L.; Nguyen, M. M.; Lee, D.; Griep, A. E.; Lambert, P. F. *J. Virol.* **2003**, *77*, 6957.
4. Koo, T. H.; Lee, J. J.; Kim, E. M.; Kim, K. W.; Kim, H. D.; Lee, J. H. *Oncogene* **2002**, *21*, 4080.
5. Chaib, H.; Rubin, M. A.; Mucci, N. R.; Li, L.; Taylor, J. M. G.; Day, M. L.; Rhim, J. S.; Macoska, J. A. *Cancer Res.* **2001**, *61*, 2390.
6. Dev, K. K.; van der Putten, H.; Sommer, B.; Rovelli, G. *Neuropharmacology* **2003**, *45*, 1.
7. Aarts, M.; Liu, Y.; Liu, L.; Besshoh, S.; Arundine, M.; Gurd, J. W.; Wang, Y. T.; Salter, M. W.; Tymianski, M. *Science* **2002**, *298*, 846.
8. Doyle, D. A.; Lee, A.; Lewis, J.; Kim, E.; Sheng, M.; MacKinnon, R. *Cell* **1996**, *85*, 1067.
9. Niethammer, M.; Valtschanoff, J. G.; Kapoor, T. M.; Allison, D. W.; Weinberg, T. M.; Craig, A. M.; Sheng, M. *Neuron* **1998**, *20*, 693.
10. (a) Toniolo, C. *Int. J. Pept. Protein Res.* **1990**, *35*, 287. (b) Gilon, C.; Halle, D.; Chorev, M.; Selinger, Z.; Byk, G. *Biopolymers* **1991**, *31*, 745.
11. Chhabra, S. R.; Khan, A. N.; Bycroft, B. W. *Tetrahedron Lett.* **1998**, *39*, 3585.
12. Yue, C.; Thierry, J.; Potier, P. *Tetrahedron Lett.* **1993**, *34*, 323.
13. (a) Leavitt, S.; Freire, E. *Curr. Opin. Struct. Biol.* **2001**, *11*, 560. (b) Ward, W. H.; Holdgate, G. A. *Prog. Med. Chem.* **2001**, *38*, 309.
14. (a) Songyang, Z.; Fanning, A. S.; Fu, C.; Xu, J.; Marfatia, S. M.; Chishti, A. H.; Crompton, A.; Chan, A. C.; Anderson, J. M.; Cantley, L. C. *Science* **1997**, *275*, 73. (b) Fuh, G.; Pisabarro, M. T.; Li, Y.; Quan, C.; Lasky, L. A.; Sidhu, S. S. *J. Biol. Chem.* **2000**, *275*, 21486. (c) Harris, B. Z.; Hillier, B. J.; Lim, W. A. *Biochemistry* **2001**, *40*, 5921. (d) Novak, K. A.; Fujii, N.; Guy, R. K. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2471.
15. Efficient Pip removal is critical, and on later occasions we found that 2–3% TFA is required. TFA levels were kept as low as possible to prevent partial release of the peptide from resin.
16. Analytical HPLC of the crude compounds showed minor peaks (attributed to multimers). Preparative HPLC that isolated the major peak in each case yielded the desired compound.