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β Strand Peptidomimetics as Potent PDZ Domain Ligands

Brief Communication

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Summary

The search for general strategies for inhibiting protein-protein interactions has been stimulated by recognition of the key role they play in virtually every process of living systems. Multiprotein complex assembly and localization by PDZ domain-containing proteins exemplify processes critical to cell physiology and function that are mediated by β strand association. Here we describe the development of substituted "@-tides," protease-resistant peptidomimetics incorporating conformationally restricted amino acid surrogates that reproduce the hydrogen-bonding pattern and side-chain functionality of a β strand. The synthetic flexibility and generality of the substituted @-tide design was demonstrated by the synthesis of a panel of ligands for the α1-syntrophin PDZ domain. The rational design of a small molecule of unprecedented affinity for the PDZ domain suggests that these peptidomimetics may provide a general method for inhibiting protein-protein interactions involving extended peptide chains.

Introduction

PDZ protein-interaction domains play a central role in a host of diverse cellular processes involving the assembly and localization of multiprotein complexes [1–3]. First discovered as the scaffolding component in synaptic signaling complexes and tight-junction assemblies, they have been identified as the recognition element in a variety of other membrane assemblies, in protein trafficking networks, and in intracellular housekeeping proteases [4]. The critical role played by PDZ domains in cellular interactions has recently been underscored by the implication that virulence of avian and human influenza strains is dictated by the spectrum of PDZ binding by viral NS1 protein [5].

PDZ domains are characterized by a cleft between an α helix and a flanking β sheet that binds a protein ligand by capturing its C-terminal tail as an extension of the β sheet (Figures 1A and 1B). Ligand specificity is thus conferred by the sequence of this C-terminal peptide, which enables the PDZ domain to bind a variety of partners independently of their overall tertiary structures. Measured dissociation constants for PDZ domain-ligand interactions are typically in the low micromolar range [6]. To date, most efforts to understand the binding properties of PDZ domains have probed sequence selectivity within peptide ligands. Library screening [7–9] and phage selection [10, 11] have demonstrated that sequences with higher affinity than the "natural" partner can be identified, and conformational constraint via side chain-side chain cyclization has led to improved binding of short peptides [12, 13]. The defined cleft in which the PDZ domain binds its ligand and the modest affinity of the complex make this protein-protein interaction potentially amenable to small-molecule inhibition [14], and, indeed, structure- and NMR-based library screening has resulted in the discovery of some non-peptidic inhibitors of PDZ domains [15–17].

Protein-protein interactions mediated by β strand association, as exemplified by the PDZ domain motif, offer many opportunities for therapeutic intervention but remain elusive targets for drug discovery. The common feature of these interactions suggests that β strand mimicry could serve as a general strategy for the design of competitive ligands for these protein binding sites. The challenge in designing a β strand peptidomimetic is to induce the extended conformation and maintain the hydrogen-bonding pattern of the peptide backbone, as well as to enable the incorporation of functionalized side chains. Designs in which covalent side chain-side chain linkages embed the peptide in a macrocyclic framework [18] or in which portions of the peptide backbone are replaced with rigid heterocyclic structures [19, 20] achieve these goals with varying degrees of success and differ considerably in synthetic accessibility. In this report, we present the development of the "aza-@-unit," **2** (Figure 1C), as a module in protease-resistant β strand mimics and describe ligands for the PDZ domain of α1-syntrophin that are significantly more potent than the corresponding peptide sequences. Since the side chains of this modular unit are incorporated from amino acids, this approach can potentially be applied to any peptide sequence.

Results and Discussion

Design and Synthesis of Substituted @-Tides

The 4,5-dihydro-2(*3H*)-pyrazinone moiety, **2**, is an enhanced design relative to the previously described 1,2-dihydro-3(6*H*)-pyridinone derivative **1** (the "@-unit") [20, 21]. Both amino acid surrogates have a cyclic structure that stabilizes the extended conformation and, when incorporated at alternating positions in a β strand peptidomimetic, also maintains a canonical hydrogen bonding pattern along one edge. We have shown previously that the simple @-unit is exceptionally effective as a stabilizing template for β-hairpin structures in aqueous solution [22]. The cyclic @-unit is easily synthesized and incorporated in oligomeric peptidomimetics, termed "@-tides" [20], but its design makes the regio- and stereoselective introduction of side chains difficult. The new aza-@-unit retains the conformational and hydrogen-bonding advantages of the original monomer and, furthermore, enables straightforward incorporation of

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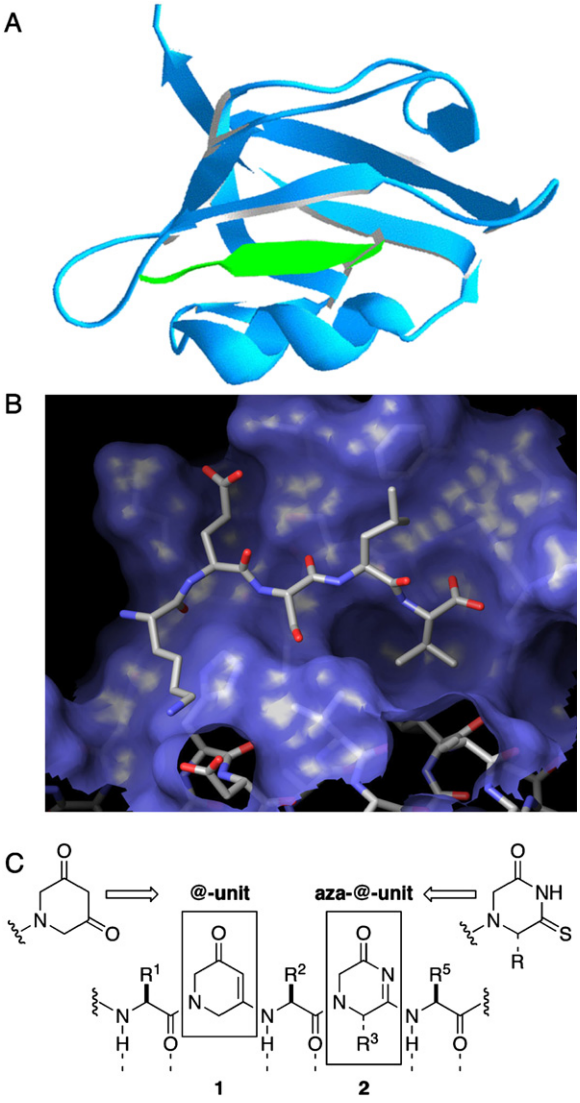

Figure 1. Extended Conformations of PDZ Ligands and @-Tides

(A) Ribbon representation of GVKESLV (green) bound to the PDZ domain of α1-syntrophin (blue).

(B) Extended conformation of the C-terminal residues of the bound peptide (both images generated from conformer 1 of 2PDZ [8]).

(C) Comparison of the unsubstituted @-unit and substituted aza-@-unit incorporated into a peptidomimetic.

side chains from amino acid precursors. @-Tides based on the substituted analogs can engage a peptide or the edge of a β sheet using the full complement of noncovalent interactions found in a natural β strand (Figure 1C).

To evaluate the efficacy and generality of this design, we synthesized a series of @-tides targeted to the PDZ domain of α1-syntrophin, based on the structure of this protein bound to a heptapeptide ligand [8]. This representative PDZ motif was chosen because it has been well characterized structurally and for its ability to bind short peptides with micromolar affinity [8]. α1-Syntrophin, a membrane-bound scaffolding protein responsible for organization of the neuromuscular junction [23], binds a variety of important protein partners through its PDZ domain, including voltage-gated sodium channels [8], neuronal nitric oxide synthase (nNOS) [23, 24], ATP binding cassette transporter (ABCA1) [25], and the α1D subtype of adrenergic receptor (AR) [26]. The ligand consensus sequence was deduced to be $(K/R)^{-4}$-E^{-3}-$(S/T)^{-2}$-X^{-1}-V^{0}, with no preference past the −4 position (following the conventional numbering system for PDZ ligands) [1]. In common with all PDZ-ligand interactions, the structure of the complex of α1-syntrophin with GVKESLV shows the backbone of the peptide to be hydrogen-bonded to the PDZ cleft at the 0, −2, and −4 positions (Val, Ser, and Lys), leaving the NH and C=O moieties at −1 and −3 (Leu and Glu) facing the exterior (Figure 1B) [8]. Thus, the glutamate and leucine residues in the peptide were the logical positions to explore the effect of @-unit replacements. To deconvolute the effect of conformational preorganization from loss of the side chain with the unsubstituted @-unit, we also included in our study peptides in which those residues were replaced with glycine or sarcosine.

The $@^{Leu}$ and $@^{Glu}$ monomers (the superscript designates the amino acid side chain on the aza-@-unit) for the construction of substituted @-tides are synthesized as cyclic monothioimides from amino acid precursors with appropriate side chain protection (Figure 2). Most conveniently, N-terminal nitrogens are protected as Cbz-derivatives, and side chains are protected with acid labile groups. Activation of the thioimide for coupling is accomplished by S-methylation with an excess of methyl iodide and one equivalent of a tertiary amine base; more strongly basic conditions lead to deprotonation at the α position and tautomerization to the unreactive, achiral 3,4-dihydro-2(*1H*)-pyrazinone. The resulting acylthioimidate couples to a peptidyl amino group in acetonitrile in the presence of ytterbium triflate as a Lewis acid catalyst, which improves dramatically the coupling efficiency and stereoretention in the 1,4-addition reaction. This catalyst also suppresses ring opening from 1,2-addition, which was observed in some coupling reactions. Once generated, the acyl amidine moiety that forms the conjugated backbone of the aza-@-unit is stable toward nonaqueous acid and base, as well as hydrogenolysis, and it is no longer susceptible to base-catalyzed tautomerization. Deprotection of the secondary amine, conventional peptide coupling, followed by repetition of the thioimide-activation and -addition steps, enables a complex peptidomimetic to be built up with alternating amino acids and aza-@-units. The modular nature of the peptidomimetic allows this synthesis to be adapted to oligomers of varying sequence and length, similar in concept to peptide synthesis.

Synthesis of KESLV peptidomimetics with $@^{Glu}$ at the −3 position was complicated by the nucleophilic nature of the Ser residue at the adjacent −2 position: deprotection of the serine hydroxyl under strongly acidic conditions (e.g., for removal of the *t*-butyl ether) causes ring opening of the dihydropyrazinone. Simple inversion of the protection strategy was stymied by the susceptibility of the benzyl ester of the $@^{Glu}$ side chain to cyclize to the lactam after deprotection of the secondary amine. An efficient alternative to acid-labile protecting groups for serine proved to be the benzyl ether. An adaptation of Sajiki's method for selective removal of benzyl carbamates effects deprotection of the N-Cbz groups while preserving the benzyl ether [27]. Final side chain deprotection after assembly of the peptidomimetic is carried

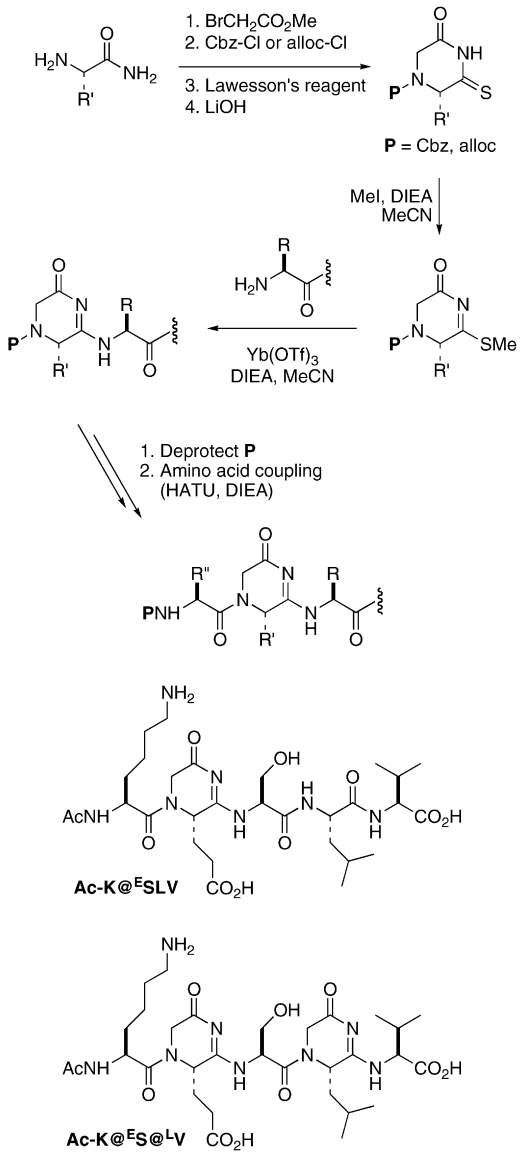

Figure 2. General Scheme for Synthesis of the Aza-@-Unit and Incorporation into a Peptidomimetic; Chemical Structures of Ac-K@^{E}SLV and Ac-K@^{E}S@^{L}V

out in two steps, with removal of acid-labile side chains with neat, anhydrous trifluoroacetic acid, followed by hydrogenolysis of the serine benzyl ether. Although some epimerization at the @^{Glu} position was observed after treatment with strong acid (3:1 mixture of S/R isomers), the diastereomers could be separated on TEA-buffered reverse-phase HPLC. The complete synthesis for Ac-K@^{E}S@^{L}V is described in the Supplemental Data (available with this article online).

Stability of Substituted @-Tides

To determine if the acyl amidine linkage of substituted @-tides is sufficiently robust to permit their use in biological applications, the stability of several derivatives was evaluated in aqueous phosphate buffer at pH 4, 7, and 10. Hydrolysis of the aza-@-unit results in loss of absorbance at 250 nm, which was monitored by UV spectroscopy. Ac-K@^{E}SLV was compared to the analog Ac-K@^{E}S(Bn)LV, which retains the serine benzyl ether, and the trimer Ac-K@^{L}V, in which the aza-@-unit is adjacent to a C-terminal carboxyl group.

The acyl amidine linkage is relatively stable in neutral or basic buffers, although labile under acidic conditions (Table 1). In neat trifluoroacetic acid, Ac-K@^{L}V undergoes cleavage of the exocyclic C-N bond to give the cyclic imide, as detected by LC-MS analysis. In contrast, treatment of Ac-K@^{E}SLV with TFA followed by exposure to water results in cleavage of the endocyclic C-N bond, as indicated by formation of an M+18 product that lacks the acyl amidine chromophore. The mechanism of this cleavage may involve transient formation of an oxazoline intermediate from attack of the serine hydroxyl, since the analogous product is not observed for Ac-K@^{E}S(Bn)LV, in which this side chain is protected. However, the similar rates at which the various @-tides undergo hydrolysis under aqueous acidic conditions suggests that all three compounds undergo hydrolysis by direct cleavage of the exocyclic C-N bond, without a significant role for proximal side chain hydroxyl or terminal carboxyl groups.

In addition to conformational control, one of the key motivations for utilizing a peptidomimetic is to reduce the vulnerability of the ligand toward proteolytic enzymes. We expected that the pyrazinone-based aza-@-unit would not fit into a protease active site because the acyl amidine linkage is so different sterically and electronically from the peptide linkage. These expectations were probed by determining the susceptibility of Ac-K@^{L}V toward cleavage by trypsin and carboxypeptidase A, in comparison to the corresponding peptide Ac-KLV. Trypsin is specific for peptide bonds at the carboxyl side of cationic amino acids (e.g., the Lys-Leu linkage), and carboxypeptidase A is specific for cleavage of C-terminal hydrophobic amino acids (e.g., the Leu-Val linkage). Their effects on the tripeptide Ac-KLV and the peptidomimetic Ac-K@^{L}V were monitored by HPLC. While both enzymes degraded the peptide rapidly, neither had any effect on the substituted @-tide over a 24 hr period. The aza-@-unit is thus inert to proteolysis at both the acyl amidine linkage as well as the tertiary amide.

Binding of Substituted @-Tides to the α1-Syntrophin PDZ Domain

The conformational restriction afforded by the @-tide peptidomimetics enhances their binding to the α1-syntrophin PDZ domain significantly relative to unsubstituted comparison peptides (Table 2). Remarkably, incorporation of @^{Glu} in place of Glu at the −3 position in Ac-KESLV increases the affinity 20-fold (entries 9 versus 1), resulting in the most potent ligand yet characterized for this protein target. The α1-syntrophin PDZ domain is known to be selective for peptides with the glutamate side chain in the −3 position, as well as more tolerant of variation at the −1 position. This selectivity can also be seen for the peptides and peptidomimetics of Table 2 as well. Whereas replacement of Glu with sarcosine abolishes binding within the range of the assay (entry 7), only a 10-fold loss in affinity results from substitution of the Leu residue with glycine or sarcosine (entries 2 and 3). The conformational constraint provided by the

Table 1. Half-Lives of Substituted @-Tides in Aqueous Solution at 20°C

		10 mM Phosphate Buffer		
Conditions:	0.1% TFA	pH 4.0	pH 7.0	pH 10.0
Ac-K@^{E}SLV	28 min	4 days	11 days	6 days
Ac-K@^{E}S(Bn)LV	31 min	4 days	23 days	15 days
Ac-K@^{L}V	29 min	2 days	15 days	12 days

Hydrolysis of the aza-@-unit was monitored by the change in UV absorbance (λ_{max} = 250 nm) at compound concentrations of ~0.2–0.3 mM. The hydrolysis reactions were analyzed as irreversible processes to determine the average half-life for each compound [30].

unsubstituted @-unit is sufficient to compensate for the loss in binding affinity from absence of the side chain at the −1 position (entry 4), but not at the −3 position (entry 8).

Additional positional effects were observed with the substituted @-tide ligands. Incorporation of the aza-@-unit is most effective at the glutamate position, where the peptidomimetic constrains the backbone to a conformation similar to that of the bound peptide. The advantage is less significant at the −1 position where the Leu residue adopts a more twisted conformation in the binding site: on replacement with @^{Leu}, the affinity is maintained (entry 6 versus entry 1) or slightly decreased (entry 10 versus entry 9). The disparate effects of rigidifying different segments of the backbone in these peptidomimetics can be rationalized from the bound conformation of GVKESLV as determined by NMR [8]. Whereas the Glu residue binds in an extended conformation, with a dihedral angle between the Lys and Glu carbonyls of 164° in the most representative of the NMR structure (model 1 from PDB entry 2pdz), the Leu backbone is twisted, with the corresponding dihedral angle of the Ser and Leu carbonyls of 129° (Figure 1B). However, even at the leucine position, where the preferred conformation of the peptide deviates from the extended conformation, substitution with the aza-@-unit is tolerated. Only incorporation of the stereoisomeric @^{DLeu} analog at this position leads to full disruption of binding (entry 5).

The substituted @-tide peptidomimetics are more effective as PDZ ligands than the corresponding unsubstituted analogs (e.g., entries 4 and 8), which confirms that both hydrogen bonding and side chain-side chain interactions are critical to successful β strand mimicry. When both the Leu and Glu residues are replaced with substituted aza-@-units (Ac-K@^{E}S@^{L}V), a modest synergistic effect is observed; that is, there is less tolerance for substitution with the @^{Leu} moiety at the −1 position when @^{Glu} replaces the glutamate at −3 (compare entry 1 versus entry 6 with entry 9 versus entry 10). Suboptimal interactions of the @^{Leu} unit are presumably accommodated less easily when the @^{Glu} moiety anchors the ligand more firmly in the binding cleft [28].

Table 2. Binding of Peptides and @-Tides to α1-Syntrophin PDZ Domain

Replacement	Entry	Ligand[a]	K_d (μM)[b]	K_d/K_d (peptide)
[Parent peptide]	1	Ac-K-E-S-L-V	6.4 ± 0.6	(1)
−1 position	2	Ac-KES-**Gly**-V	70 ± 6	11
	3	Ac-KES-**Sar**-V	61 ± 4	9.5
	4	Ac-KES-@-V	15 ± 2	2.3
	5	Ac-KES-@^{DL}-V[c]	>100	>15
	6	Ac-KES-@^{L}-V	7.7 ± 0.4	1.2
−3 position	7	Ac-K-**Sar**-SLV	>100	>15
	8	Ac-K-@-SLV	>100	>15
	9	Ac-K-@^{E}-SLV	0.32 ± 0.01	0.05
−1 and −3 positions	10	Ac-K-@^{E}-S-@^{L}-V	1.0 ± 0.1	0.16

Binding to purified α1-syntrophin PDZ domain was assayed in 50 mM HEPES buffer (pH 7.5), 50 mM NaCl at 25°C in competition with a dansylated peptide (Dns-SIESDV, K_d = 2.1 ± 0.1 μM [9]). Dissociation constants greater than 100 μM were not adequately defined by this assay and thus are reported as a lower limit. Dissociation constants ≤1 μM were calculated by a modified mathematical analysis to remove the assumption that competitor ligand is always in large excess relative to the fluorescent ligand or protein used in the assay (see Supplemental Data).

[a] Syntheses of peptides and unsubstituted @-tides were performed on solid support following procedures described previously [21]. The substituted @-tides were synthesized as shown in Figure 2 and detailed in the Supplemental Data.

[b] The dissociation constants for the peptidomimetics were determined relative to that measured concurrently for the peptide Ac-KESLV and normalized to the average K_d value of the latter. The dissociation constant determined for the pentapeptide Ac-KESLV is similar to those reported for longer, nonacetylated peptides with the same C-terminal sequence [8, 9].

[c] This compound incorporates the D-enantiomer of the @^{Leu} unit.

Significance

With a K_d value of 0.32 μM, Ac-K@^{E}SLV is bound more tightly to the α1-syntrophin PDZ domain than one of its extensively studied protein ligands, nNOS; the dissociation constant for this protein is 0.61 μM [9], although it contacts the PDZ domain over a greater surface area than the peptidomimetic and with additional residues in the interfacial β strand [24]. Indeed, Ac-K@^{E}SLV is one of the most tightly bound small-molecule ligands reported for any PDZ domain. The highest affinity PDZ ligand of which we are aware is WETWV, selected as a ligand for the Erbin PDZ domain by phage display; Skelton et al. report an IC_{50} value of 0.020 μM for this peptide from a bead-based fluorescence assay [29]. Although the doubly substituted analog Ac-K@^{E}S@^{L}V is slightly weaker than Ac-K@^{E}SLV, it is nonetheless more tightly bound than the parent peptide from which it was designed. Because of the ability of the aza-@-unit to mimic an extended peptide residue in conformation, side chain functionality, and hydrogen bonding along one edge, and because of the resistance of oligomeric @-tides to proteolysis, they show considerable promise as β strand peptidomimetics. Substitution of selected residues in extended peptides with the corresponding aza-@-units is a strategy for enhancing binding affinity that may be applied to ligands for other PDZ domains and to a variety of protein-protein interactions mediated by β strand association.

Supplemental Data

Supplemental Data include general materials and methods; protease assays; synthesis of Cbz-@$^{Glu(OtBu)}$ monothioimide; synthesis of Ac-K@^{E}S@^{L}V, **10**; characterization data for other assayed compounds; and the "no assumptions" method for calculating

dissociation constants. They are available at http://www.chembiol.com/cgi/content/full/13/12/1247/DC1/.

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