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Peptide Chemical Ligation Inside Living Cells: In Vivo Generation of a Circular Protein Domain

Julio A. Camarero[a,†], David Fushman[b,‡], David Cowburn[b] and Tom W. Muir[a,*]

[a]*The Laboratory of Synthetic Protein Chemistry, The Rockefeller University, New York, NY 10021, USA*
[b]*Laboratory of Physical Biochemistry, The Rockefeller University, New York, NY 10021, USA*

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Abstract—Here we describe the first example of a peptide chemical ligation reaction performed inside a living cell. A cell-based native chemical ligation approach was developed and used to generate a circular version of the N-terminal Src homology 3 (SH3) domain from the murine c-Crk adapter protein inside *Escherichia coli* cells. The in vivo cyclization reaction was extremely efficient and the resulting circular protein domain was fully biologically active and able to adopt the native SH3 folded structure. This work represents an important step towards the in vivo generation of small backbone cyclic peptides for use in basic biological research.

Introduction

Many pharmaceutically important natural products, including several antibiotics and immuno-suppressants, are based on cyclic peptides. Indeed, peptide cyclization is commonly used in medicinal chemistry for modifying the properties of bioactive peptides.[1–3] In particular, backbone (head-to-tail) cyclization has been widely used to rigidify peptide structure, therefore minimizing the entropic cost of receptor binding, and to improve the in vivo stability of peptides. The chemical synthesis of cyclic peptides has been well explored and a number of solution-based and solid-phase strategies are now available for this purpose.[4–6]

There is currently a great deal of interest in developing biosynthetic routes to novel cyclic peptides. Important progress towards this goal has been made in two areas, namely, non-ribosomal peptide synthesis[7] and expressed protein ligation/protein trans-splicing.[8–12] The former strategy involves genetically engineering non-ribosomal peptide synthetases and is reminiscent of the more established technologies for generating novel polyketides.[13] The later strategy relies on heterologous expression of recombinant peptides fused to modified intein protein splicing elements.[14] In principle, both approaches provide a way to genetically encode the chemical structure of the final cyclic peptide.

Access to backbone cyclic peptides using recombinant DNA expression techniques offers the intriguing possibility of producing large combinatorial libraries of drug-like molecules using the tools of molecular biology. Such an approach would be analogous to peptide phage-display technology,[15] but would have the advantage of producing stable backbone cyclic peptides rather than linear peptides or disulfide-based cyclic peptides. The approach would differ from peptide phage display in another important respect; a backbone cyclic peptide, by definition, cannot be covalently linked through its mainchain to another protein. Thus, in order to permit genetic encoding of the cyclic peptide library, each member must remain inside the cell that produced it and the biological selection/screening assay must also take place in vivo. Consequently, a key feature of this

Abbreviations: CBD, chitin binding domain; EDTA, ethylenediaminetetraacetic acid; ESMS, electrospray mass spectrometry; HSQC, heteronuclear single quantum correlation; IPTG, isopropyl-1-thio-α-D-galactopyranoside; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; MAP, Methionyl aminopeptidase; SH3, Src homology 3; TFA, trifluoroacetic acid. Standard IUPAC single and triple letter codes for amino acids are used throughout.

*Corresponding author. Tel.: +1-212-327-7368; fax: +1-212-327-7358; e-mail: muirt@rockvax.rockefeller.edu
†Current address: Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, University of California, Livermore, CA 94550, USA.
‡Current address: Center of Biomolecular Structure & Organization, University of Maryland, College Park, MD 20742-3360, USA.

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technology would be the ability to generate a cyclic peptide inside a living cell.

Benkovic and co-workers recently described a clever split intein approach for generating backbone cyclized polypeptides in vivo.[10] This technology exploits the observation that protein splicing can occur in *trans* when N- and C-terminal intein fragments are reconstituted.[16–18] Protein trans-splicing provides a means of linking two polypeptides (referred to as the N- and C-exteins) together simply by fusing the exteins to the appropriate end of the complementary intein fragments. In the cyclization system, the peptide to be cyclized is nested between two fragments of the naturally occurring *Ssp* DnaE split intein[19] such that the C-terminal intein fragment is fused to the N-terminus of the peptide and vice versa. Protein splicing leads to generation of the desired backbone cyclized polypeptides inside *Escherichia coli* cells. A potential limitation of this approach is the apparent requirement for specific N- and C-extein residues at the intein junctions in order for efficient protein splicing to occur[12]—this may limit the sequence diversity within the cyclic peptide.

As an alternative route to the in vivo generation of backbone cyclized peptides, we wondered whether it would be possible to perform an intramolecular version of the Kent's native chemical ligation inside cells. Native chemical ligation relies on the chemoselective reaction between an N-terminal Cys residue in one peptide and an α-thioester group within a second peptide, resulting in the formation of a normal peptide bond.[20] It is now well established that cyclization occurs when both these reactive groups are incorporated into a synthetic peptide precursor.[21–24] Moreover, approaches are now available that allow the two reactive groups needed for ligation to be introduced into recombinant proteins (reviewed in ref 25). Thus, an N-terminal cysteine can be introduced by cleaving (by proteolysis or autoproteolysis) an appropriate precursor fusion protein, whereas an α-thioester group can be incorporated by thiolysis of a corresponding intein-fusion protein. Using these approaches, the in vitro synthesis of circular versions of recombinant peptides and proteins has been possible.[8,9,11]

An intriguing aspect of the intramolecular native chemical ligation reaction is that it can occur in the absence of thiol co-factors[8]—this contrasts with the intermolecular reaction where thiols are always required for efficient ligation.[26] These thiol-independent cyclization reactions take place when the N- and C-termini of the linear peptide precursor are juxtaposed, either because of the short length of the peptide or because of the topology of a native protein fold.[8,23] Indeed, we have shown recently that a cyclic recombinant SH3 domain can be prepared from the corresponding intein-fusion simply by unmasking the N-terminal cysteine in the domain; that is, cyclization occurs in the absence of thiols.[8] Importantly, this result suggests that the cyclic SH3 domain will also be generated in vivo provided that an N-terminal cysteine is present in the expressed fusion protein.

The initiating methionine residue is removed from most of the cytosolic proteins in *E. coli*. This post-translational modification is carried out by the endogenous enzyme methionyl amino-peptidase (MAP) and the efficiency of this processing event is strongly dependent upon the nature of the residue adjacent to the Met.[27] Fortunately, Met-Cys motifs are processed relatively efficiently. Indeed, Iwai and Pluckthun recently used this cell processing event to introduce an N-terminal Cys into a β-lactamase-intein fusion.[11] This allowed a cyclic version of β-lactamase to be prepared in vitro.

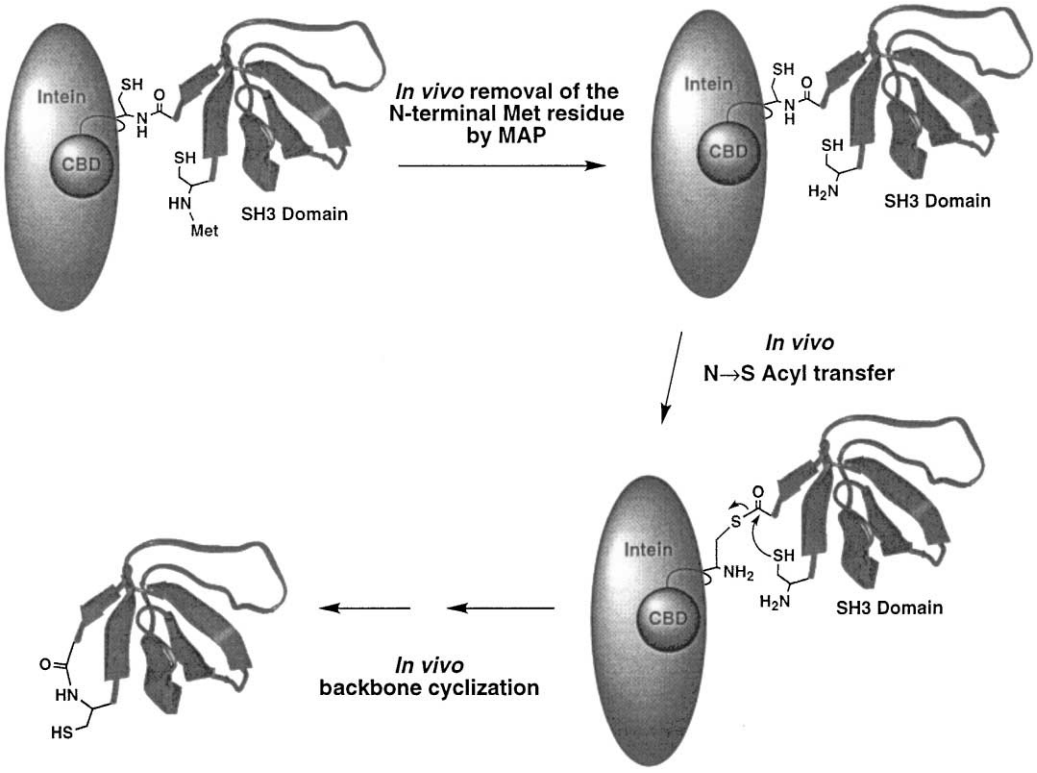
In the present study, the above observations have been integrated into a cell-based intramolecular native chemical ligation approach (Scheme 1). This was used to generate a circular version of the N-terminal Src homology 3 (SH3) domain from the murine c-Crk adapter protein inside *E. coli* cells. The in vivo cyclization reaction was extremely efficient and the resulting circular protein domain was fully biologically active and able to adopt the native SH3 folded structure. To our knowledge, this is the first example of a peptide chemical ligation reaction performed inside a living cell and, importantly, represents an important step towards the in vivo generation of small backbone cyclic peptides for use in basic biological research.

Results

As a demonstration of our in vivo cyclization approach, we attempted to generate a backbone cyclized version of the N-terminal SH3 domain from the c-Crk adaptor protein inside *E. coli* cells. This 57-residue protein domain possesses a globular structure composed of five β-strands which position the N- and C-termini in close proximity.[28] In a previous communication, we showed that a backbone cyclized SH3 domain could be very efficiently prepared in vitro from a recombinant linear precursor.[8] In that case we used a combination of intein-technology, which allowed the introduction of an α-thioester group, and a mutagenesis/factor Xa proteolysis strategy, which allowed the controlled introduction of an N-terminal Cys upon in vitro proteolysis.

In order to carry out the cyclization reaction entirely inside the cell, we constructed a bacterial expression plasmid in which the gene corresponding to the SH3 domain (residues Y^{136} to Y^{190} of murine c-Crk) was cloned in frame to an engineered VMA intein. The fusion protein was modified at the DNA level in order to introduce an N-terminal Met-Cys-Gly- motif as well as an extra Gly residue at the C-terminus of the SH3 coding region. The Gly residues were introduced to facilitate the cyclization reaction as well as to stabilize the new loop formed in the product.[29]

We anticipated that efficient in vivo removal of the Met residue from the Met-Cys-Gly- motif by endogenous MAP coupled with facile backbone cyclization, would allow the desired circular SH3 to be biosynthesized inside *E. coli* cells (Scheme 1). This was indeed found to be the case. SDS-PAGE analysis of the soluble cell



Scheme 1. In vivo biosynthesis of circular SH3 protein domain. The first step involves the expression of the SH3-intein-CBD fusion protein (CBD refers to the chitin binding domain affinity handle). Importantly, the amide bond connecting the SH3 domain to the intein is in equilibrium with an α-thioester (i.e., the intein promotes an N→S acyl transfer). Shortly after being expressed, the endogenous methionine-aminopeptidase (MAP) cleaves the N-terminal Met from the expressed fusion protein yielding an N-terminal Cys residue. The presence of both an N-terminal Cys residue and an α-thioester leads to a spontaneous intramolecular native chemical ligation to give the cyclic SH3 domain.

fraction, revealed that most of the SH3 domain had been cleaved from the intein in vivo (Fig. 1A, lane ii). It is conceivably, that the remaining SH3-intein fusion protein still contained the initiating Met residue and thus could not participate in the intramolecular ligation reaction. Unfortunately, we were unable to isolate enough of this material to test this hypothesis. Remarkably, when the entire soluble cell fraction was analyzed by reversed-phase HPLC, the expected circular protein and the cleaved intein-CBD were found to be the major components in the mixture (Fig. 1B). It is worth noting that we were unable to detect any of the linear SH3 domain, suggesting that in vivo hydrolysis of the α-thioester linkage present in the precursor was minimal. The circular SH3 domain was purified from the cellular supernatant as described in the methods section and characterized by tryptic digestion and ESMS (Fig. 1B, see insert). The isolated yield of cyclic protein was estimated to be around 2 mg/L.

A fluorescence-based binding assay was used to compare the ligand-binding properties of the cyclic SH3 domain with the linear version of the protein, prepared as previously described.[8] As predicted from the structure of the SH3 domain, cyclization did not have any deleterious effect on the ligand-binding activity. On the contrary, the cyclic domain showed a slightly higher affinity for the peptide ligand than the linear SH3 domain ($K_{d,cyclic} = 0.47 \pm 0.04$ μM, $K_{d,linear} = 0.90 \pm 0.06$ μM).

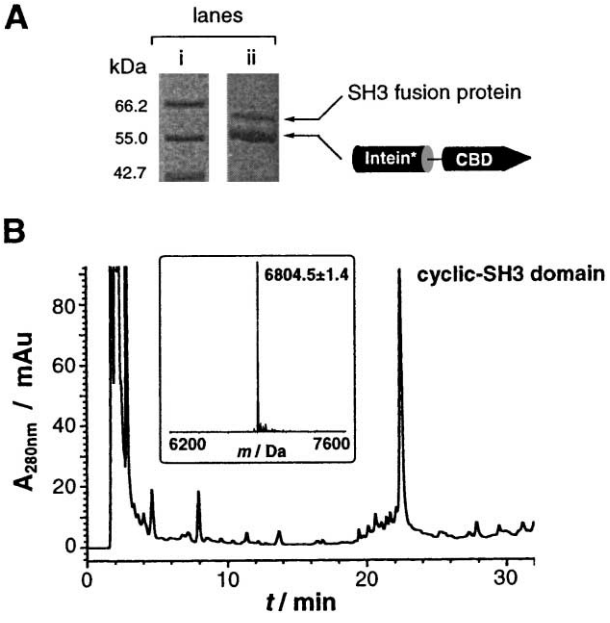


Figure 1. The in vivo cyclization reaction is extremely efficient. (A) SDS-PAGE analysis of; lane i, protein markers; lane ii, affinity purified intein-fusion proteins recovered from the soluble cellular extract. (B) Analytical reverse-phase HPLC trace of the soluble cellular extract. Note, in the chromatogram shown (gradient = 30–45% B over 30 min.), the intein-CBD cleavage product elutes in the column wash. Inset: ESMS of the purified circular SH3 domain.

Heteronuclear NMR, spectroscopy was used to compare the solution structure of the cyclic and linear SH3 domains. Note, this required the preparation of uniformly ^{15}N-labeled samples (see Methods). As can be seen in Fig. 2, the ^{1}H{^{15}N} HSQC spectra obtained for the cyclic and linear SH3 domains were very similar, indicating that both topological versions of the SH3 share the same global fold. In both cases, the resolution and dispersion were indicative of a well-defined solution structure with a significant portion of its backbone in a β-conformation.[28] A more detailed description on the structural properties of the cyclic SH3 domain is beyond the scope of this communication and will be published elsewhere.

Discussion

Native chemical ligation is an established approach for the total synthesis[30] and semi-synthesis[25] of proteins. The approach relies on a chemoselective reaction between an α-thioester and an N-terminal cysteine and results in the formation of a normal peptide bond. These two reactive groups can either be in different polypeptides (intermolecular ligation) or in the same polypeptide (intramolecular ligation/cyclization). Native chemical ligation is an extremely robust technique and can be performed in the presence of a wide variety of additives including, thiols, various organic solvents, chemical denaturants, detergents and other proteins.[30] Indeed, these reactions have recently been performed with peptides fused to bacterio-phage coat proteins.[31]

In the present study, we have demonstrated that an intramolecular version of native chemical ligation can occur in an environment as complex as the cytosol of a living bacterial cell. This technique has been used to prepare multi-milligram amounts of a cyclic version of the Crk-SH3 domain. This in vivo ligation reaction was found to be remarkably clean and efficient; the majority of the intein fusion protein underwent the expected cleavage event based on SDS-PAGE analysis of the cell lysate. Moreover, only the cyclic version of the SH3 protein was detected in the soluble fraction using a combination of HPLC and mass spectrometric analysis. This can be understood in terms of the high effective molarity of the two reactive groups caused by the native structure of the SH3 domain (the N- and C-termini are ~8 Å apart). It is possible that competing reactions, for example hydrolysis or thiolysis of the thioester, will be more prevalent in cases where the termini are not so close together. Consequently, this in vivo approach might be most useful for topologically predisposed proteins or for relatively short peptides that are known to undergo efficient macrocyclizations. Future studies will address these issues in more detail.

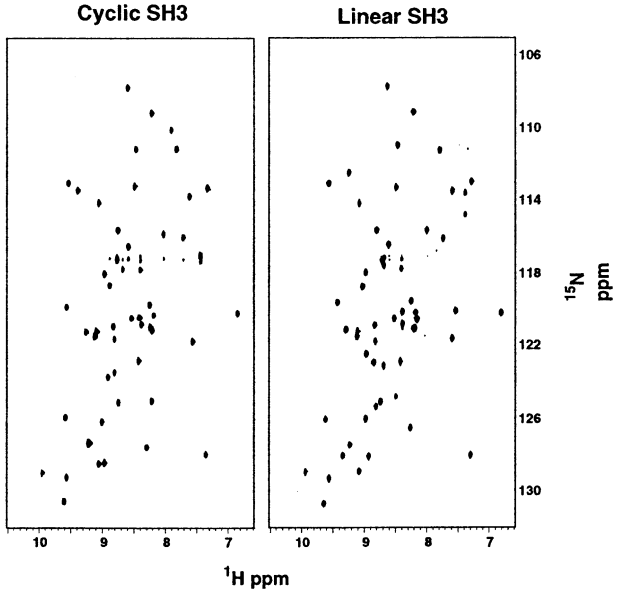


Figure 2. The cyclic SH3 domain has a native-like fold. Shown are the amide regions of the 2D ^{1}H{^{15}N} HSQC NMR spectra at 500 MHz for the cyclic and linear SH3 constructs.

We were also able to prepare multi-milligram quantities of uniformly ^{15}N-labeled circular Crk-SH3 for solution NMR studies. Preliminary analysis of the ^{1}H{^{15}N} HSQC spectra obtained for the circular and linear SH3 domains reveals that the backbone constraint does not perturb the global fold of the protein. This is consistent with the ligand-binding studies, which if anything suggest that the circular protein binds ligand slightly more tightly than the linear protein. These findings are not entirely surprising given the topology of the Crk-SH3 native fold and the fact that short linker introduced in the circular protein should minimize any strain induced by the constraint. A detailed account of the thermodynamic, kinetic and structural consequences of backbone cyclization in this system will be described elsewhere.

Our in vivo native chemical ligation approach requires a cellular processing event to generate the requisite N-terminal Cys for ligation. In the present work, this involved cleavage of a Met-Cys motif by endogenous methionyl aminopeptidase. Although this reaction was very efficient in the example described, it may not be work for all peptides—conceivable the identity of residues downstream of the Cys might influence the cleavage efficiency. It is worth noting, however, that it should also be possible to introduce an N-terminal Cys into a cellular protein using either an engineered intein [9] or a ubiquitin-fusion strategy.[32]

One of the attractions of the existing split-intein cyclization system [10,12] is that it is not reliant on a cellular proteolysis event, rather it involves an in-built protein trans-splicing reaction. The approach exploits the naturally occurring *Ssp* DnaE split intein and has been used by Benkovic and co-workers to prepare a circular peptide and a circular protein in vivo.[10] More recent studies indicate that a small number of native N- and C-extein residues (~ 5 residues in total) are required for efficient protein trans-splicing to occur using the *Ssp* DnaE split intein[12] (interestingly, neither of the circular molecules prepared in the original study contained native extein

residues). Conceivably, this could restrict the sequence diversity in the circular peptide. The only absolute sequence requirement for native chemical ligation is an N-terminal cysteine; model studies have shown that all 20 natural amino acids can support ligation when located at the C-terminus of the peptide α-thioester.[33] Moreover, the engineered inteins used to generate recombinant peptide α-thioesters are compatible with most amino acids upstream of the cleavage site.[14] Thus, our in vivo native chemical ligation approach may be quite general with respect to the sequence of the linear peptide precursor.

In summary, we have described a new strategy to generate head-to-tail cyclic polypeptides in vivo. A cell-based native chemical ligation reaction was used to generate a circular version of the N-terminal Src homology 3 (SH3) domain from the murine c-Crk adapter protein inside *E. coli* cells. To our knowledge, this is the first example of a peptide chemical ligation reaction performed inside a living cell. As originally noted by Benkovic and co-workers,[10] the ability to prepare cyclic polypeptides in vivo could have enormous utility in basic biological research. For example, it may be possible to prepare combinatorial libraries of small drug-like cyclic peptides and screen them for their ability to attenuate cellular processes. In addition, in vivo backbone cyclization should improve the thermodynamic and proteolytic stability of a protein and thus could be used to increase the cellular half-life of a protein.

Experimental

General materials and methods

Analytical gradient HPLC was performed on a Hewlett-Packard 1100 series instrument with 214 and 280 nm detection. Analytical HPLC was performed on a Vydac C18 column (5 micron, 4.6×150 mm) at a flow rate of 1 mL/min. Preparative HPLC was performed on a Waters DeltaPrep 4000 system fitted with a Waters 486 tunable absorbance detector using a Vydac C18 column (15–20 micron, 50×250 mm) at a flow rate of 50 mL/min. All runs used linear gradients of 0.1% aqueous TFA (solvent A) versus 90% acetonitrile plus 0.1% TFA (solvent B). Electrospray mass spectrometry (ESMS) analysis was routinely applied to all synthetic peptides and components of reaction mixtures. ESMS was performed on a Sciex API-100 single quadrupole electrospray mass spectrometer. Calculated masses were obtained using the program MacProMass (Sunil Vemuri and Terry Lee, City of Hope, Duarte, CA). Expressed proteins were routinely analyzed on SDS-PAGE using the standard procedures.

Tryptic digestion

50 μg of purified circular protein was dissolved in 200 μL of 20 mM Tris HCl, 5 mM EDTA buffer at pH 8. Lyophilized trypsin (10 μg, sequencing grade, Sigma) was then added. The digestion was allowed to proceed for 3 h at 37 °C before being quenched by addition of 20 μL TFA and analyzed by HPLC. The tryptic fragments were collected and analyzed by ESMS.

In vivo biosynthesis of circular SH3 3

The DNA encoding the c-Crk N-SH3 domain (residues Y^{136}–Y^{190} of murine c-Crk protein) was isolated by PCR from a cloned c-Crk gene (kindly provided by Professor H. Hanafusa, RU). The 5′ primer (5′-G ATT CTC AGG CAG CAT ATG TGC GGC TAT GTG CGG GCC CTG TTT G-3′) encoded a NdeI restriction site as well as Met and Cys codons, fused in frame with the N-terminus of the SH3 domain. The 3′ oligonucleotide primer (5′-AC TGA GGC GGA GGC AGG TAC GTA CTT CTC CAC GTA AGG GAC-3′) created the SnaBI restriction site and introduced a Gly codon fused in frame with the C-terminus of the SH3 domain. The PCR-amplified SH3 domain was purified, digested simultaneously with NdeI and SnaBI and then ligated into the NdeI-SmaI-treated plasmid pTYB2 (New England Biolabs). The resulting pTYB2-MC-SH3 plasmid was shown to be free of mutations in the c-Crk SH3-coding region by DNA sequencing. Six liters of *E. coli* BL21 cells transformed with pTYB2-MC-SH3 plasmid were grown to mid-log phase ($OD_{600}\approx0.6$) and then induced with 0.5 mM IPTG for 4 h at 37 °C. After centrifugation, cells were resuspended in lysis buffer (0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium phosphate, 500 mM NaCl buffer at pH 7.5, containing 5% v/v glycerol) and lysed using a French press. The lysate was clarified by centrifugation at 14,000 rpm for 30 min. The supernatant was loaded on a C18 cartridge (2.5 g silica-C18, Sep-Pak Waters), extensively washed with water (5×10 mL) and finally eluted with MeCN/water (7:3 v/v, 5×10 mL). The organic washes were pooled, the MeCN removed in vacuo and the circular SH3 domain purified by preparative HPLC using a linear gradient 30–45% B over 45 min. The pure material was characterized by tryptic digestion and ESMS as the desired circular material [Observed Mass = 6804.3 ± 1.3 Da; expected (av. isotope comp.) 6806.4 Da]. The isolated yield of circular protein was estimated to be around 2 mg/L.

Cloning and expression of linear SH3 domain

The linear SH3 domain (residues A134 to Y190) was expressed using a Glutathione-*S*-Transferase fusion strategy as previously described.[8]

Expression of uniformly labeled ^{15}N SH3 domains

Uniformly ^{15}N labeled SH3 domains were obtained by growing the corresponding transformed BL21 *E. coli* cells in M9 minimal medium, supplemented with 0.2% glucose and 0.1% $^{15}NH_4Cl$ (99% enriched). The M9 was also supplemented with 100 mg/L ampicillin and 5 mg/L thiamin hydrochloride. The expression conditions were identical to those employed using Luria-Bertani medium. The expression yields using M9 medium were between 60–70% of those obtained with Luria–Bertani medium.

Fluorescence-based ligand binding assays

The affinity constants of the circular and linear versions of the SH3 domain for peptide ligand (sequence; PPPALPPKKRXYX, where X stands for 6-aminohexanoic acid) were measured using a fluorescence-based titration assay. Experiments were conducted at 25° C in a stirred 1 cm-pathlength cell using a Spex Fluorolog-3 spectrofluorimeter. Excitation was at 298 nm with a 5 nm slit and the fluorescence emission was monitored at 355 nm through a 5 nm slit. In all cases, the protein concentration was kept at 0.5 μM in a buffer containing 40 mM sodium phosphate, 150 mM NaCl at pH 7.2. The dissociation constants were determined by changes in the fluorescence of the protein solution upon addition of ligand at defined concentrations; calculations were made assuming formation of 1:1 complex and fitting, by nonlinear least-squares analysis (GraphPad Prism v2.0a), the corrected fluorescence intensities to the equation:

$$F = \frac{F_P + [L] \times F_{PL}/K_d}{1 + [L]/K_d}$$

where F_P corresponds to the fluorescence in absence of ligand, F_{PL} is the fluorescence of the protein-ligand complex, K_d is the dissociation constant and $[L]$ is the free peptide ligand concentration. All the experiments were performed in triplicate. Protein and peptide concentrations were determined by UV absorption; SH3 linear and circular domains ($\lambda = 280$ nm), 15400 M^{-1} cm^{-1}; peptide ligand ($\lambda = 276$ nm), 1345 M^{-1} cm^{-1}.

NMR spectroscopy

NMR samples were prepared by dissolving the corresponding protein in a buffer containing 20 mM sodium phosphate, 20 mM DTT-d_{10} 100 mM NaCl at pH 7.2, containing 10% D_2O and 0.1% NaN_3. The protein concentration in each case was around 1 mM. Heteronuclear $^1H\{^{15}N\}$ HSQC experiments were performed on a Bruker DMX-500 spectrometer at 31 °C.

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