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NMR structure note

Solution structure of a novel C_2-symmetrical bifunctional bicyclic inhibitor based on SFTI-1

Agnès M. Jaulent[a], Arnd B. E. Brauer[a,b], Stephen J. Matthews[c] & Robin J. Leatherbarrow[a,*]

[a]*Department of Chemistry, and* [c]*Department of Biological Sciences, Imperial College London, South Kensington Campus, London, SW7 2AZ, UK;* [b]*Institut für Chemie und Biochemie, Freie Universität Berlin, Otto-Hahn-Bau, Thielallee 63, D-14195 Berlin (Dahlem), Germany*

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Abstract

A novel bifunctional bicyclic inhibitor has been created that combines features both from the Bowman–Birk inhibitor (BBI) proteins, which have two distinct inhibitory sites, and from sunflower trypsin inhibitor-1 (SFTI-1), which has a compact bicyclic structure. The inhibitor was designed by fusing together a pair of reactive loops based on a sequence derived from SFTI-1 to create a backbone-cyclized disulfide-bridged 16-mer peptide. This peptide has two symmetrically spaced trypsin binding sites. Its synthesis and biological activity have been reported in a previous communication [Jaulent and Leatherbarrow, 2004, PEDS 17, 681]. In the present study we have examined the three-dimensional structure of the molecule. We find that the new inhibitor, which has a symmetrical 8-mer half-cystine CTKSIPP′I′ motif repeated through a C_2 symmetry axis also shows a complete symmetry in its three-dimensional structure. Each of the two loops adopts the expected canonical conformation common to all BBIs as well as SFTI-1. We also find that the inhibitor displays a strong and unique structural identity, with a notable lack of minor conformational isomers that characterise most reactive site loop mimics examined to date as well as SFTI-1. This suggests that the presence of the additional cyclic loop acts to restrict conformational mobility and that the deliberate introduction of cyclic symmetry may offer a general route to locking the conformation of β-hairpin structures.

Abbreviations: 1D – One-dimensional; 2D – Two-dimensional; BBI – Bowman–Birk inhibitor; BiKK – cyclo[$(CTKSIPPI)_2$] with disulfide bridge; NOESY – Nuclear Overhauser effect spectroscopy; RMSD – Root mean square deviation; SFTI-1 – sunflower trypsin inhibitor-1, cyclo(CTKSIPPICFPDGR) with disulfide bridge; TOCSY – Total correlation spectroscopy

Biological context

The Bowman Birk family of inhibitors is a collection of small serine protease inhibitors that are found mainly in plant seeds (see McBride et al. (2002) for a review). The BBIs are small proteins (7–9 kDa) containing a high number of disulfide bridges (seven), and typically have two symmetrical homology domains. Each domain presents a loop that projects out of the main protein core, thus making BBIs resemble "bow ties" (Chen et al., 1992). The projecting loops incorporate the

*To whom correspondence should be addressed. E-mail: r.leatherbarrow@imperial.ac.uk

reactive site of the inhibitor, and are the only part in direct contact with the protease upon binding. Each reactive site is a 9-mer loop delimited by a disulfide bridge. It was shown early on that the inhibitory properties are largely unaffected by removal of the main protein core following partial digestion (Odani and Ikenaka, 1973; Nishino et al., 1977), which indicated to researchers that synthetic BBI mimics would most probably be biologically active. Since then, numerous synthetic BBI mimics have indeed been synthesised and tested (see McBride et al. (2002) for a review). The consensus is that synthetic reactive loops retain the BBIs' inhibitory properties providing the structural disulfide bridge is present, making the minimum motif for a BBI mimic a 9-mer, disulfide-enclosed loop. We have shown that this disulfide-enclosed reactive loop represents an independent β-hairpin structural motif that is conserved in isolation (Brauer et al., 2001), providing a rationale for the potency of the mimics.

The same structural β-hairpin motif is also found in SFTI-1, a small peptidic protease inhibitor isolated from sunflower seeds (Luckett et al., 1999). With only 14 residues, SFTI-1 is to date the smallest yet most potent of all BBIs, illustrating that a large size is not necessary for potent inhibition. SFTI-1 displays an atypical structure; it contains the usual disulfide-cyclized 9-mer reactive loop of the BBIs, but this is not built onto a proteinaceous core as is the case for BBI proteins, but rather onto a second, small non-reactive loop made of 5 residues. The whole sequence is therefore head-to-tail cyclized. It is the combination of these two loops that makes SFTI-1 such a potent inhibitor (Luckett et al., 1999; Jaulent et al., 2001; Korsinczky et al., 2001). However, unlike the larger BBI proteins, since SFTI-1 possesses only a single reactive loop, it is unable to inhibit two proteases simultaneously (Jaulent and Leatherbarrow, 2004).

With this difference in inhibitory valency in mind, we have designed bifunctional inhibitors that aimed to combine favourable features from both inhibitors; on the one hand, to possess the ability of the BBI proteins for simultaneous dual inhibition, and on the other hand, to be small and constrained in a similar way to SFTI-1 (See Figure 1 of Jaulent and Leatherbarrow, 2004). Two novel inhibitors were constructed; one that has a completely symmetrical sequence with each of the two reactive sites directed towards trypsin, and one that incorporates two distinct activities and separately targets trypsin and chymotrypsin. In this communication, we report the solution structure of the bifunctional trypsin inhibitor, which we refer to as BiKK, as elucidated by NMR spectroscopy.

Methods and results

Experimental

The BiKK inhibitor was synthesised and purified as previously described (Jaulent and Leatherbarrow, 2004). The NMR sample was obtained by dissolving the BiKK peptide in a 90% H_2O/10% D_2O solvent containing 3-trimethylsilyl-1-propanesulfonic acid as an internal reference. The concentration of the peptide was 2 mM and the pH* adjusted to 3.8.

1D, standard TOCSY (mixing time of 80 ms) and NOESY (mixing time of 250 ms) experiments were run at 305 K on a Bruker AMX 600 spectrometer and further processed and analysed using the SpinWorks 2.1 (Dr. K. Marat, University of Minnesota) and MestRe-C (Cobas and Sardina, 2003) software packages. Water suppression was performed using a WATERGATE scheme. Due to an unusually good signal to noise ratio, no further experiments were needed to complete the analysis. Assignment was achieved using a standard sequential assignment method (Wüthrich, 1986). All coupling constants were derived from 1D spectra.

Structure calculation was carried out with the TINKER software suite (Hodsdon et al., 1996). The program DISTGEOM was used to refine model structures against the NMR-derived restraints with the distance geometry, simulated annealing, and energy minimization protocol that has been described in detail in the original publication using the AMBER force field (Hodsdon et al., 1996). In all calculations, distance restraints were generated based on cross-peak volumes, calibrated against the distance of the NOE of the two β methylene protons of proline (1.78 Å), and were implemented with a lower limit of 1.86 Å and upper limits of 2.40, 2.85, 3.35 or 5.00 Å, with a difference of at least 0.30 Å between the upper limit and the NOE-derived distance. For methyl protons an upper limit of up to 7.00 Å was used. ϕ-Restraints were implemented as $-120° \pm 20°$,

$-120° \pm 45°$ and $-70° \pm 5°$ for residues with $^3J_{HNH\alpha}$ coupling constants ≥9.9 Hz, ≥7.1 Hz, and proline, respectively; an upper limit of $-100°$ was implemented for serine residues in order to avoid ϕ-dihedral angles which do not reflect the experimentally observed $^3J_{HNH\alpha}$ coupling constant. On the basis of diagnostic sequential NOE connectivities (Wüthrich, 1986), ω-dihedral angles were restrained to either 180° or 0° with limits of ±6°. Experimentally determined preferred χ^1 rotamers were implemented with limits of ±30° for non-proline residues and ±15° for proline residues as described previously (Brauer et al., 2003). No hydrogen bond restraints were used in the structure calculations. From one of the calculation runs, a set of 20 structures with the lowest energy were selected as a family of structures, the coordinates of which have been deposited in the PDB under the ID code 2BEY.

Structure of the BiKK inhibitor: Analysis of the 1D NMR spectra

The amide region of the one-dimensional spectrum for the BiKK inhibitor is of particular interest as it reveals several structural properties of the inhibitor (Figure 1). The BiKK inhibitor is a 16-residue peptide, though this includes 4 proline residues that do not give rise to amide proton resonances. While this leaves 12 amide protons that might be expected to give rise to doublets, only 6 such resonances are observed in the amide region of the spectrum. The design of the BiKK inhibitor is such that the sequence is effectively that of an 8-mer half-cystine loop CTKSIPP′I′, repeated in a C_2 symmetrical fashion. The presence of only 6 unique amide proton resonances therefore proves that the solution structure is also symmetrical, as there is perfect superimposition of the resonances for pairs of corresponding protons within the two identical sequence segments.

Further analysis of the 1D spectrum shows evidence that the BiKK inhibitor is highly structured. The amide protons have a wide dispersion of chemical shifts, with values ranging from 7.3 to 8.9 ppm. Mostly, the amide proton chemical shifts are shifted downfield compared to "random coil" values (Wüthrich, 1986; Wishart et al., 1991). All signals within the spectrum display very narrow resonance lines. This allowed coupling constants to be determined directly from the one-dimensional spectrum. The $^3J_{HNH\alpha}$ coupling constants, in their majority, were found to be >8 Hz. Most $^3J_{H\alpha H\beta}$ coupling constants were either <4 Hz or >10 Hz. Taken together, these results indicate that both symmetrical parts of the peptide adopt a well-defined, β-type conformation (Wishart et al., 1991).

A further feature of this spectrum that distinguishes it from that found for many related BBI-derived inhibitory loops is that there is no detectable evidence of conformational heterogeneity. In many BBI-mimics we find a significant proportion of minor conformational isomers that represent *cis*/*trans* isomers about one or both proline residues (Brauer et al., 2001; Brauer et al., 2002b; Brauer et al., 2003). These minor conformers are manifest by the presence of small peaks in the spectrum—in favourable cases we have been able to assign these and even calculate their structure (Brauer et al., 2002a). However, the spectra of BiKK exhibit no such heterogeneity, suggesting that no significant level of slow *cis*/*trans* isomerization is found amongst the four proline residues of the sequence.

The solution structure of BiKK

Figure 2 shows two views of a same family of 20 lowest-energy structures for the BiKK inhibitor, calculated from the analysis of the 1D and 2D NMR data (see supplementary material for the corresponding statistics). Over the region of the peptide backbone, there is remarkably good overlap amongst the family of structures (RMSD 0.24 ± 0.08 Å). Together with the evidence presented earlier, it seems clear that the BiKK inhibitor is extremely well structured in solution. We presume that this results from the previously noted propensity of these sequences to adopt a β-hairpin conformation (Brauer et al., 2001) in conjunction with the constraints imposed by the bicyclic structure.

The most striking feature of the overall structures is their symmetry, which is immediately obvious in each view, with the C_2 symmetry axis itself located between the two sulfur atoms of the disulfide bridge.

Structure of the 9-mer reactive loop

Each disulfide-enclosed loop is equivalent in structure and adopts a well-defined type VI β-turn for the SIPP part. A strong diagnostic $H\alpha_i$-$H\alpha_{(i+1)}$ NOE signal between Ile and Pro establishes

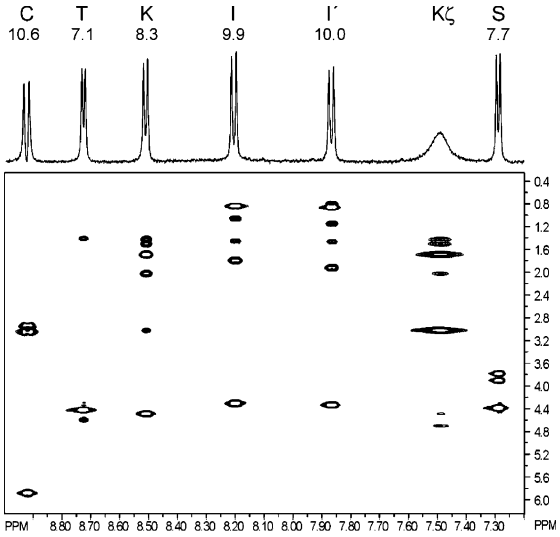

Figure 1. Amide protons region of the 2D TOCSY spectrum recorded for a sample of BiKK (90% H_2O/10% D_2O). The signals corresponding to the one-dimensional HN region are shown as a band at the top of the two-dimensional TOCSY plot. The HN resonances are labelled with the single letter amino acids code and the values of the corresponding $^3J_{HNH^{\alpha}}$ coupling constants in Hertz are indicated.

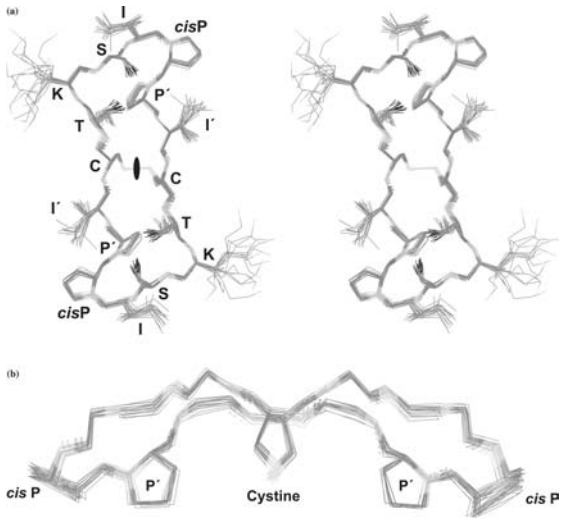

Figure 2. Superimposition of 20 lowest energy structures for the BiKK inhibitor. a. A stereo view of the family of BiKK structures shown with all heavy atoms and side chains, with the superimposition being performed relative to the backbone atoms. The two β-hairpin structures are seen to be symmetrical around the disulfide bridge, at the centre of which the location of the C_2 symmetry axis is indicated. b. Side-view of the same family of structures. For clarity, only the backbone, the proline residues and the disulfide bridge are shown.

this peptide bond to be *cis*, whilst two strong $H\alpha_i$-$H\delta/\delta'_{(i+1)}$ NOE signals between Pro and Pro' shows this latter to be *trans* (Wüthrich, 1986). There is a bulge on the loop that exposes the P1 residue. This particular arrangement is characteristic of canonical inhibitors (Bode and Huber, 1992).

The chirality of the disulfide bridge could not be verified experimentally as no inter-residual NOEs could be determined for the participating cysteine residues due to their symmetry-induced identical chemical shifts. However, the observed $-60°$ χ^1 rotamer for both cysteine residues results in a short right-handed hook conformation (Richardson, 1981; Srinivasan et al., 1990) for the disulfide bridge in the calculated structures, which is consistent with that observed in BBI proteins and BBI mimics (Brauer et al., 2002b).

Discussion and conclusions

Structure of BiKK

We have shown in a preceding communication that the bifunctional BiKK peptide does indeed function as a trypsin inhibitor (Jaulent and Leatherbarrow, 2004).

The novelty and originality of the BiKK structure lies in its true symmetry, which is in contrast to the pseudo symmetry found for the BBI proteins. The biological activity studies for the BiKK inhibitor indicate that not only does the BiKK peptide function as a trypsin inhibitor, but each inhibitory loop also acts independently and equivalently (Jaulent and Leatherbarrow, 2004). This is consistent with the structural data that shows each loop to be able to adopt the required canonical conformation, demonstrating that the disulfide bridge is effectively shared without loss of its structural role towards either loop. Although the design scheme made the sequence symmetrical, symmetry of conformation might not necessarily have followed. As BiKK is very small and constrained, this could have resulted in distortion in solution from the canonical conformation of at least one of the two loops or of the disulfide bridge, therefore abolishing symmetry of the structure. The NMR data, however, clearly confirm that each canonical loop has the same structure.

BiKK displays exceptional rigidity

An unusual feature of the BiKK NMR spectra is the absence of any detectable peaks from minor

conformers arising from *cis*/*trans* isomerization of the proline peptide bonds. These populations of minor conformers are found in many BBI loops that we have studied, where they do not usually exceed 20% of the total signal intensity, with the dominant conformer exhibiting a *cis* conformation for the Xaa-Pro bond (P2′–P3′) (Brauer et al., 2002a). However, for BiKK, exclusively *cis* conformers about the Ile-Pro bond were detected. It is likely that the additional constraints imposed by the head-to-tail cyclization and the potential steric clashes in alternative conformations have resulted in the observed conformation being the only one that is significantly populated. This total lack of conformational heterogeneity is unprecedented in any other BBI-mimics for which solution structures have been reported to date.

Conformation of the reactive loop

Figure 3 shows an overlay of the backbone structures of the BiKK inhibitor with SFTI-1 (a) and with an 11-mer BBI-mimic (b). The superimpositions were done over the reactive loop region. From these superimpositions, it can be seen that the overlay for the reactive loops of BiKK and SFTI-1 on the one hand, and BiKK and the 11-mer BBI loop on the other hand are both very good. Since all loops exhibit the required conformation for inhibition, it is not surprising that all peptides are potent, nanomolar (or lower) inhibitors of trypsin. These inhibitors only differ in their head-to-tail cyclization, or lack thereof. The good overlay shows that the reactive loop conformation is barely affected by changes outside the disulfide-linked region.

It has been shown that the biological activity of a BBI mimic is mediated through conformations having the *cis* Xaa-Pro (P2′–P3′) only (Brauer et al., 2002a), with the *trans* form not detectably interacting with the target protease. NMR studies on the 11-mer loop showed the presence of a minor conformer population (less than 20%) that was attributed to the *cis*-*trans* proline equilibration (Brauer et al., 2002b). A similar situation is found for SFTI-1 (Korsinczky et al., 2001), where the authors reported minor conformer populations of up to 40%. We have also recorded NMR spectra of SFTI-1 (data not shown) that confirm this observation, although we find a slightly lower proportion of minor conformers. It therefore seems that the presence of the second symmetrical

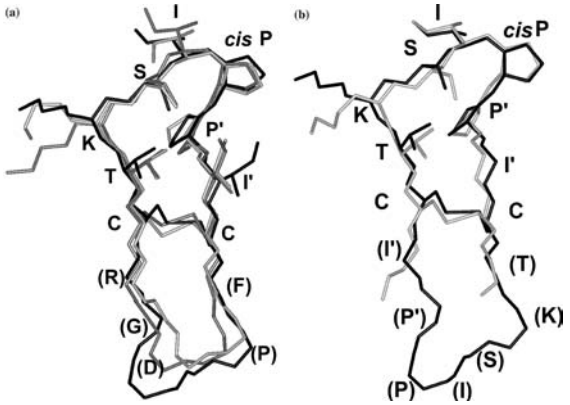

Figure 3. Superimposition of the CTKSIPP′I′C loop from the BiKK inhibitor with SFTI-1 and with an 11-mer BBI mimic. a. BiKK (black sticks) is superimposed with the two structures available for SFTI-1: the crystal structure (dark grey sticks, 1SFI from Luckett et al. (1999)), and the ^{1}H NMR structure (light grey sticks, 6JBL from Korsinczky (2001)). b. BiKK (black sticks) is superimposed with the antitryptic 11-mer BBI mimic (light grey sticks, 1GM2 from Brauer et al. (2002b)). The residues are labelled using brackets to denote the second loop of SFTI-1 (a) and the BiKK inhibitor (b).

loop in the non-natural BiKK structure has a more beneficial effect on defining the loop conformation than occurs in the natural SFTI-1 inhibitor. We should again note, however, that SFTI-1 displays better potency than the BiKK inhibitor does (Jaulent and Leatherbarrow, 2004).

SFTI-1 and BiKK

As was the intention, SFTI-1 and BiKK have very similar reactive site structures. Both inhibitors are bicyclic; their backbone is cyclic, and a disulfide bridge further delimits two loop regions. The first region for both inhibitors is a 9-mer, disulfide bridged reactive loop bearing the exact same sequence. The difference between the two inhibitors lies in their second region; in the BiKK inhibitor, we have replaced the non-reactive, 5-mer turn of SFTI-1 by a second, reactive 9-mer loop. By doing so, we hoped to achieve bifunctionality, whilst retaining the conformational advantages conferred upon SFTI-1 by its atypical structure. The superimposition of the two reactive loops from the two inhibitors is very good (RMSD over the backbone 0.45 Å).

Although truly bifunctional, the small size of BiKK does not allow it to bind to two trypsin molecules simultaneously (Jaulent and Leatherbarrow, 2004). Therefore, when one reactive loop from the BiKK is bound to trypsin, the second

reactive loop plays only a "passive" role. As the binding of BiKK to trypsin is slightly better than it is for an isolated 11-mer loop, it is possible that stability is added to the inhibitor-trypsin complex *via* additional non-covalent interactions and/or entropic advantages associated with the more highly defined conformation. The increased rigidity provided by the second loop may also be the reason for the improved stability of BiKK against hydrolysis by trypsin. The half-life time of hydrolysis for the first reactive loop of the intact BiKK was found to be about twice as long as that of both an isolated 11-mer loop and the BiKK with one loop already cleaved (Jaulent and Leatherbarrow, 2004).

The superimposition of the two inhibitors' respective backbones over their non-binding regions shows a good fit for the first residues immediately following the disulfide bridge, before the structures necessarily deviate. As SFTI-1 is a natural inhibitor, it is reasonable to speculate that the residues in the non-reactive site loop have been optimised by evolution to maximize their interactions when in complex with the protease. For example, it has been found that SFTI-1's arginine (P4) side chain makes contact with the Asn97 of trypsin (Luckett et al., 1999). The equivalent position in BiKK is occupied by an isoleucine residue, and it is therefore probable that an advantageous interaction with the enzyme is lost in the BiKK-trypsin complex. Furthermore, SFTI-1 forms a hydrogen bond between the side chain of its aspartate residue in the non-reactive site loop and the side chain of Gln175 in trypsin (Luckett et al., 1999); again this interaction will be lost in the BiKK inhibitor as the equivalent position is occupied by a proline. These additional interactions are therefore likely to be amongst the reasons why SFTI-1 binds approximately 60 times more tightly to trypsin than BiKK does.

Conclusions

In a previous communication, we presented the design, synthesis and biological activity of novel bifunctional bicyclic inhibitors based on the BBI and SFTI-1 (Jaulent and Leatherbarrow, 2004). We have now elucidated the solution structure of the bifunctional trypsin inhibitor, BiKK. Our results show that each of BiKK's reactive loops adopts the classical canonical conformation typical of small serine proteinase inhibitor proteins, as well as of all synthetic BBI mimics we have structurally analysed. We have also shown that the overall structure of the inhibitor is symmetrical, with a C_2 symmetry axis situated between the two sulfur atoms of the shared disulfide bridge. A particularly remarkable finding is that BiKK proves to be exceptional amongst BBI mimics in showing for each one of its two symmetrical loops a unique structural identity unmarred by the presence of minor, biologically inactive conformers. This contributes to making BiKK a potent trypsin inhibitor.

As it appears that the engineered symmetry widens the energetic gap between the desired and potential alternative conformations, we suggest that deliberate introduction of such symmetry could be a useful instrument in the molecular designer's tool box.

Electronic supplementary material is available at http://dx.doi.org/10.1007/s10858-005-1210-9.

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