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Circular β-lactamase: stability enhancement by cyclizing the backbone

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Abstract We have cyclized the polypeptide backbone of β-lactamase with a short peptide loop as a novel method for protein stabilization, using intein-mediated protein ligation. Successful cyclization was proven by mass spectrometry and subsequent relinearization by proteolytic cleavage, as well as by resistance against carboxypeptidase. Under the conditions of the experiment, no disulfide bond is present. The circular form of β-lactamase was found to be significantly more stable against irreversible aggregation upon heating than the linear form. The circular form could be purified from the linear one either by this heat treatment or by a his-tag which became exopeptidase-resistant by cyclization. The increased stability of the circular form is probably due to the decreased conformational entropy in the unfolded state and in the intermediate states. While the introduction of additional disulfide bonds for protein stabilization follows the same rationale, the cyclization strategy may disturb the structure less and thus constitute a general method for stabilizing those proteins with N- and C-termini in close proximity.

Key words: Circular protein; Protein splicing; Intein; β-Lactamase; Protein stability

1. Introduction

The engineering of stable proteins is of great technological and economic importance, since the limited stability of proteins often severely restricts their medical and industrial application. The stepwise improvement by a combination of rationally designed mutations or evolutionary approaches is, however, still a major undertaking and challenge for every individual protein. The engineering of novel disulfide bonds has been one of the more frequently used strategies to stabilize proteins. In natural proteins, disulfide bonds can make substantial contributions to the stability [1,2] and part of this effect is presumed to be due to the decrease of the conformational entropy in the unfolded state [3]. However, more detailed treatments of enthalpic and entropic contributions point to a more complicated picture, which is protein dependent [4,5]. Importantly, because of the short reach and hydrophobicity of the disulfide bond, it often has to be engineered in the interior of the protein, such that the new disulfide bond frequently interferes with the remainder of the structure. Despite great successes in several cases, an increase in stability has not been routinely achieved [6,7]. Furthermore, additional disulfide bonds usually complicate the production of the protein.

An alternative way to reduce the conformational entropy in the unfolded state is the cyclization of the polypeptide backbone. Cyclization of peptides is commonly used to reduce their conformational flexibility, thereby locking them in an active conformation (for review, see [8]). It has been shown that the cyclization of peptides frequently improves their in vivo stability and biological activity [9]. In contrast to peptides, the cyclization of proteins has not been much explored. Goldenberg and Creighton produced the first circular protein from bovine pancreatic trypsin inhibitor (BPTI), a small protein of 58 amino acids, by chemical cross-linking of the backbone termini with a peptide bond [11]. They have found no significant stabilization effects by the backbone cyclization, however, and the characterization of the physicochemical properties of other cyclized proteins has been limited [10,12–14]. The better characterization of cyclic peptides than proteins is also due to the fact that cyclic peptides and proteins have usually been prepared by well established chemical methods in vitro, which cannot easily be applied to larger proteins. A general method to produce a circular form of peptides and proteins from a bacterial expression system would open new avenues for the physicochemical characterization of such circular proteins and their subsequent application in biotechnology.

Such a general method of cyclization is provided by the protein splicing reaction. During the course of these studies, two other reports using this approach have appeared [13,14]. Protein splicing is a self-catalyzed post-translational modification, in which a precursor protein is self-spliced into two polypeptides by excising the protein splicing domain called intein, or alternatively protozyme, and joining the flanking protein sequences (N-extein and C-extein) (Fig. 1c) [15,16]. The key chemical step, catalyzed by the intein, is an acyl migration, leading to the formation of a thioester from a peptide bond (Fig. 1e), which can thus be attacked by nucleophiles. A partial reaction of a modified intein, in which the active site was mutated from HNC to HAC, is also used for protein purification [17], where the N-extein can be cleaved off with nucleophiles such as DTT from the intein, which is immobilized by a fused chitin binding domain (CBD).

Inteins can also catalyze a *trans*-ligation. Two different strategies have been used for this purpose. In one approach, the intein itself has been split and the two halves of the intein have been re-assembled in vitro, each fused to a different extein [18]. In another approach, an intein domain has been used, carrying the aforementioned HNC to HAC mutation in the catalytic site, such that the reaction can be triggered with a thiolate nucleophile such as DTT and subsequently, an external peptide containing a N-terminal cysteine can be linked as depicted in Fig. 1a [19]. These reactions are very promising for segmental-specific isotope labelling, as required in NMR for solving the structures of larger proteins [20]. This protein ligation utilizing inteins has been called expressed protein

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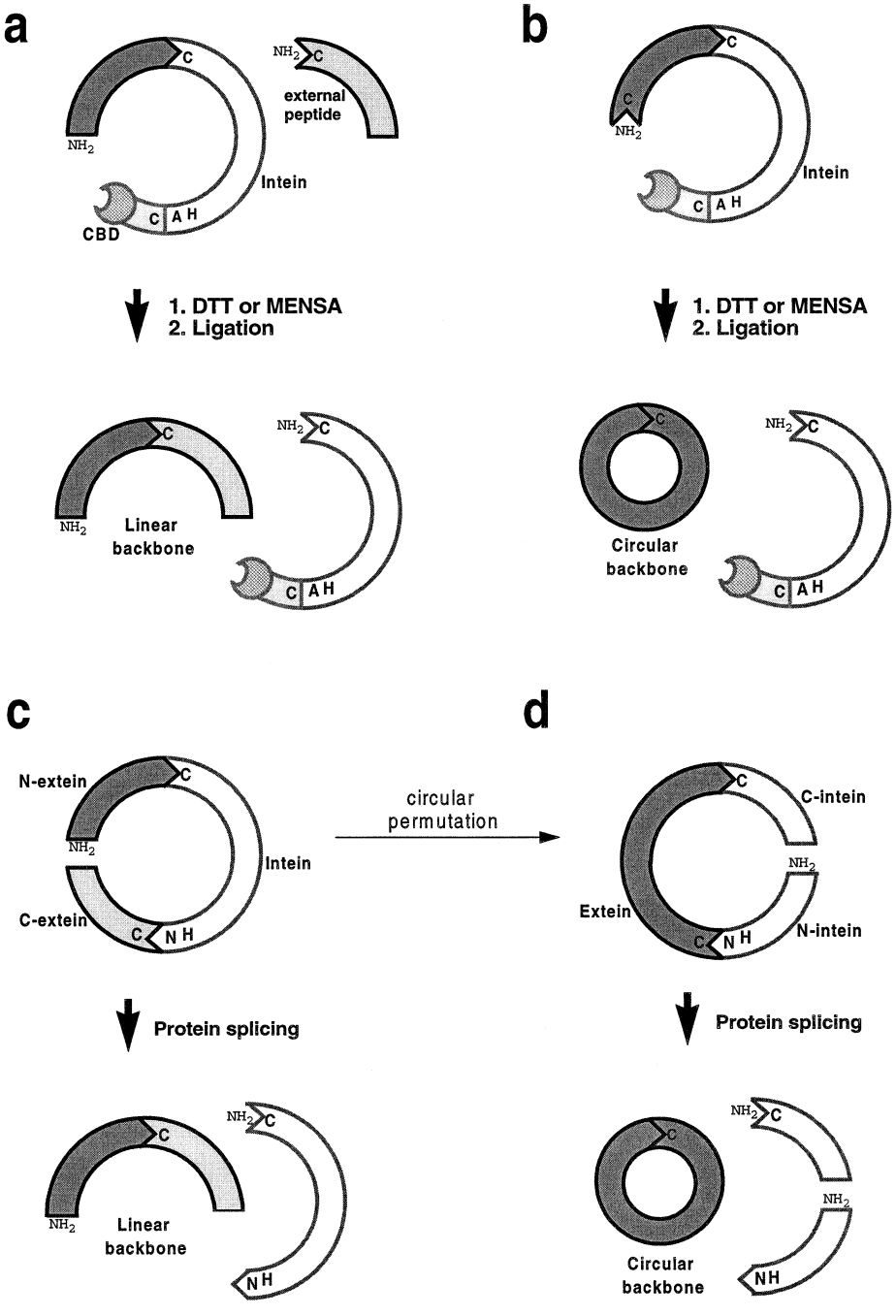
ligation (EPL) or intein-mediated protein ligation (IPL) [21,22].

Both types of ligation approaches can in principle be used to make circular proteins (Fig. 1b and d). In its simplest form, only a cysteine residue needs to be introduced at the N-terminus, which will lead to cyclization, provided that N- and C-termini can reach each other (Fig. 1b). In the second approach, a circular protein can in principle be produced by a circular permutation of the precursor protein containing an intein domain (Fig. 1d). This approach would have the advantage that it would require only the formation of the three-dimensional structure and does not require other factors such as thiolates, making it in principle possible to produce circular peptides and proteins totally in vivo. Both approaches may open a new general strategy to produce cyclic peptides and proteins from a bacterial expression system.

In the present paper, we concentrate on the first approach and we report the production of a 30 kDa protein, β-lactamase (BLA), with a circular topology, in which the N- and C-termini are cross-linked with a normal peptide bond (Fig. 2). The circular protein was made from an *Escherichia coli* expression system by making use of the enzymatic activity of an intein domain. Under the conditions of the experiments, the disulfide bond of BLA is not present. We verified the circular nature of the product by three independent methods, purified the circular form by two different methods and determined that the circular form of BLA is more stable than the linear form upon heating, as would be expected from polymer theory.



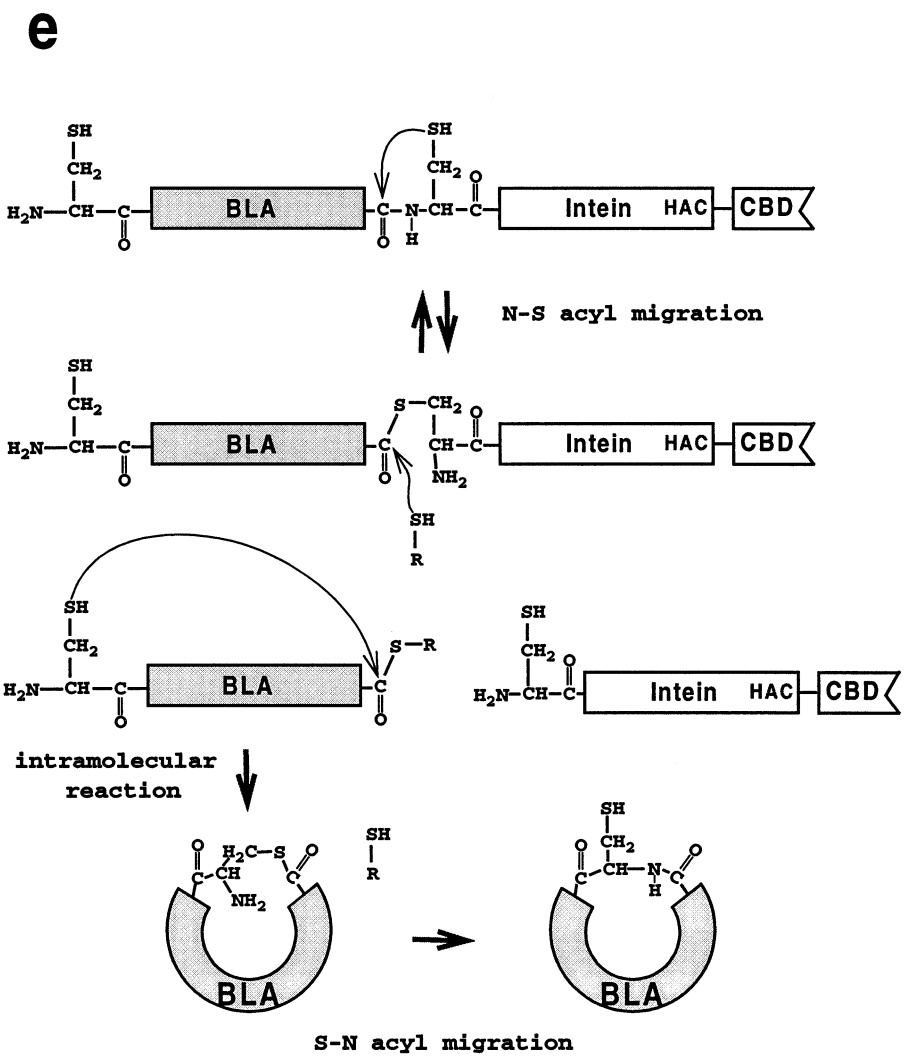


Fig. 1. Schematic representation of (a) the EPL with a modified intein [21,22]. The active site of the intein is modified from HNC to HAC so that the protein splicing activity is abolished, but the C-terminus of the N-extein domain can be cleaved by sulfur nucleophiles and at the same time converted to a thioester, which allows for the subsequent ligation reaction with an external peptide carrying a N-terminal cysteine. (b) Ligation reaction as in scheme (a), except that the nucleophilic N-terminal cysteine is located in the same protein. The ligation reaction results in a circular peptide backbone. (c) Ligation of two extein domains by protein splicing. This is the natural reaction of intein-mediated protein splicing with the wild-type active site residues HNC. (d) Circular permutation of the precursor protein containing an intein domain with a wild-type active site as in scheme (c). Protein splicing results in a circular protein, with the original N- and C-termini connected by a peptide bond. (e) The simplified mechanism of the cyclization reaction. The precursor protein with a N-terminal cysteine undergoes N-S acyl migration. The intra-molecular thioester can be attacked by thiolate nucleophiles such as MENSA, resulting in cleavage of the fusion protein, carrying now another C-terminal thioester. The released protein with the thioester can react with a N-terminal cysteine either in an intra-molecular or inter-molecular reaction.

2. Materials and methods

2.1. Expression plasmids of TEM-1 BLA

The gene of TEM-1 BLA was amplified from the plasmid pGEX-2TK (Pharmacia) with the following two oligonucleotides: 5′-GGATGCATATGTGTGCTAGCGGTGGTGGTCACCCAGAAACGCTGG-3′ and 5′-CGAATTCCCCGGGTCCCCAATGCTTAATCAG-3′. The PCR product was cloned into the vector pTYB2 (New England Biolabs) between the *Nde*I and *Eco*RI sites, resulting in the plasmid pT2R31E. The mature TEM-1 BLA so obtained has a N-terminal linker, CAGGGG, and a C-terminal linker, GPGEFLEPG, fused to the *Sce* VMA gene (whose active site Asn was replaced with Ala and linked to the CBD) [17] (Figs. 1b and 3). Three other plasmids were derived from this plasmid by replacing the C-terminal sequence of BLA between the *Eco*RI and *Kpn*I sites with synthetic oligonucleotides, yielding the expression plasmids for BLA-His, BLA-Trb and BLA-S as shown in Fig. 3. The DNA sequences of all constructs were confirmed with PCR cycle sequencing.

2.2. Cyclization of BLA

The proteins were typically expressed in 2 l bacterial culture of *E. coli* ER2566 (New England Biolabs) [22], grown at 22°C. The culture was induced with 0.5 mM isopropyl-thio-galactoside at an OD_{600} of 0.7, growth was continued for 6 h and the cells were harvested, lysed by sonication and subsequently by a French press. The cell lysate was cleared by centrifugation at 15 000 rpm with an SS34 rotor and then loaded on a chitin column (15 ml) (New England Biolabs). After washing with loading buffer (50 mM Tris pH 8.0, 0.1 mM EDTA, 0.1% Triton X-100, 500 mM NaCl), the chitin beads were briefly washed with the cyclization buffer (0.2 M NaCl, 0.2 M sodium phosphate, pH 5.0) and then stirred in 15 ml cyclization buffer, containing 50 mM 2-mercaptoethanesulfonic acid (MENSA), overnight at room temperature. The supernatant from the beads was collected, which contains the circular and linear BLA released from the fusion protein. As the protein is produced in the cytoplasm and kept under reducing conditions, the single disulfide bond of BLA does not form.

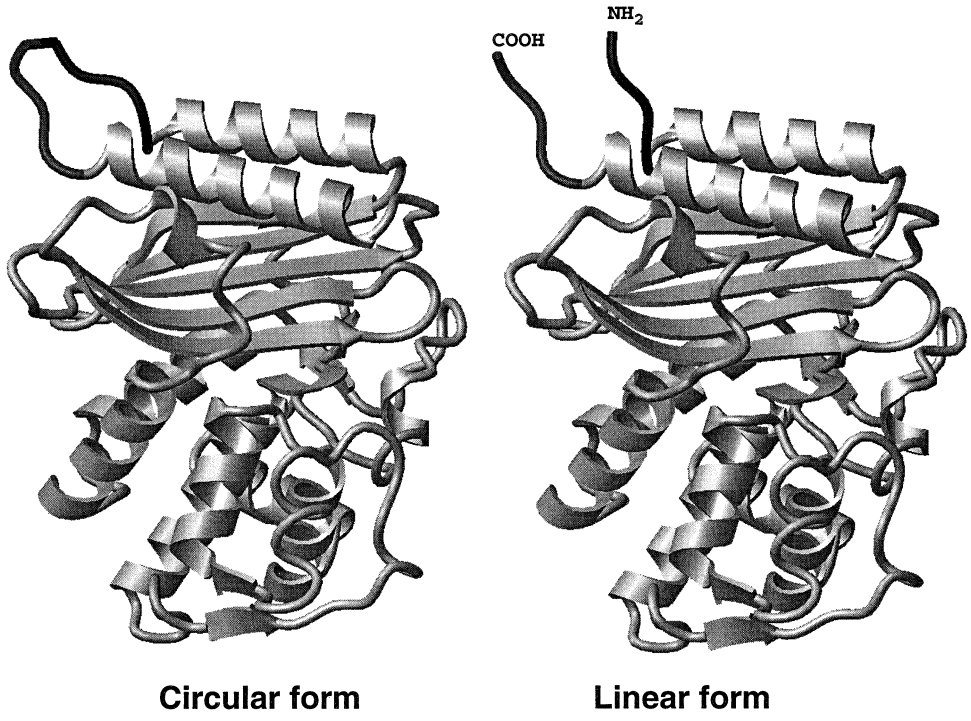


Fig. 2. Models of the three-dimensional structure of circular and linear BLA (BLA-S). The models were created based on the coordinates of the PDB entry 1AXB with MOLMOL [31,32]. NH_2 and COOH denote N- and C-termini, respectively, in the linear form. The introduced N-terminal linker is colored black and the C-terminal linker is colored dark gray.

2.3. Stability test of circular and linear BLA

The stability against heat precipitation was quantitatively tested as follows. Two-hundred μl of a 40 μM protein solution, directly taken from the ligation reaction in the reaction buffer (200 mM NaCl, 200 mM NaPi, 50 mM MENSA, pH 5.0) was incubated at various temperatures (from 40 to 70°C). The insoluble fraction was removed by centrifugation at 20 800×*g* for 30 min. The protein pellets and the supernatant, precipitated with 5% TCA, were dissolved with sodium dodecyl sulfate (SDS) loading buffer. Each soluble and insoluble fraction was analyzed with SDS-polyacrylamide gel electrophoresis (PAGE). After Coomassie brilliant blue staining, the individual bands were quantified with a densitometer (Model 300A, Molecular Dynamics).

2.4. Linearization of circular BLA

The supernatant from the cyclization reaction of BLA-Trb, in which a thrombin-specific cleavage site was introduced (Fig. 3), was incubated at 46°C for 1 h in order to selectively precipitate most of the linear form of BLA-Trb. The insoluble fraction was removed by centrifugation and the buffer was changed to 50 mM Tris, 1 mM EDTA, pH 7.0, with a Microcon concentrator (Amicon). The circular protein was incubated for 2 h at room temperature with 0.01 U of thrombin (Roche Diagnostics).

3. Results

We chose TEM-1 BLA (wild-type 263 amino acids) as a model protein for cyclization since its activity and three-dimensional structure as well as its stability properties have been well characterized [23–25]. Its N- and C-termini are in close proximity in the three-dimensional structure (Fig. 2). We introduced a cysteine residue after the start codon, which becomes the N-terminal residue, as the starting methionine is usually removed, if cysteine is the following residue [28]. Five additional amino acids were added behind the N-terminal cysteine in front of the BLA sequence, while nine residues were fused to its C-terminal end, followed by the modified *Sce* VMA gene, itself fused to a CBD [17] (Figs. 1e and 3). After cyclization, the original N- and C-termini of BLA would then be connected by a loop of an additional 15 amino acids (Figs. 2 and 3). Three other variants were constructed, in which the sequence of the C-terminal linker of BLA was modified for alternative purification and analytical procedures (Fig. 3, see Section 2). The proteins were expressed in *E. coli* and the cyclization reaction was performed in vitro, after the protein was bound to the chitin column and the column was washed. The chitin beads were incubated with 50 mM MENSA overnight at room temperature in order to cleave the fusion protein and simultaneously to obtain a thioester at the C-terminus, followed by the cyclization reaction as depicted in Fig. 1e. SDS-PAGE analysis of the reaction mixture indicated two major closely spaced bands with an apparent molecular weight of about 30 kDa. We now provide evidence that the more slowly migrating one is the linear form of BLA, while the faster migrating one is the circular form.

The successful cyclization of the protein was confirmed by several independent methods. First, both bands in SDS-PAGE were analyzed by N-terminal Edman degradation

	β-lactamase		Intein
	Linker	w.t. β-lactamase	Linker
BLA	MCASGGG**HPE**.....**KHW**GPGEFLEPG-CFAK...		
BLA-S	MCASGGG**HPE**.....**KHW**GPGEFG----CFAK...		
BLA-His	MCASGGG**HPE**.....**KHW**GPGEF**HHH**A-CFAK...		
BLA-Trb	MCASGGG**HPE**.....**KHW**GPGEF**LVPRG**CFAK...		

Fig. 3. Amino acid sequences of the linkers at the N- and C-termini of TEM-1 BLA. The linker sequences are underlined. The methionine start codon, which gets cleaved during biosynthesis, and part of the sequence from *Sce* VMA are also shown. The his-tag and the thrombin site are indicated in bold (see text).

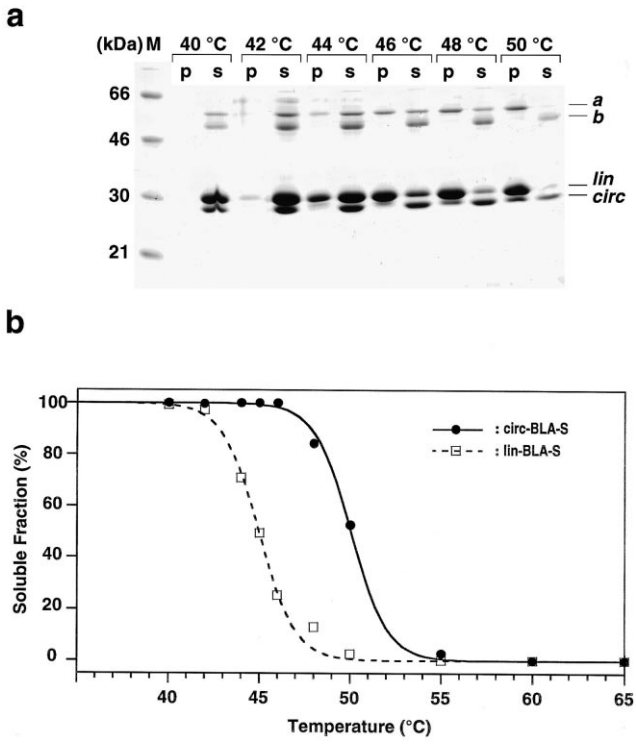


Fig. 4. (a) SDS-PAGE analysis of the soluble and insoluble fraction after 1 h of incubation at different temperatures. *s* and *p* denote soluble fraction and insoluble fraction, respectively. *lin* and *circ* indicate the linear form and the circular form, respectively. For bands *a* and *b*, see text. (b) Plot of the soluble fraction of monomeric BLA-S after 1 h incubation at different temperatures. Filled circles and open rectangles indicate the circular and the linear form of BLA-S, respectively. A midpoint of the transition of 45°C was obtained for the linear form and 50°C for the circular form.

and amino acid analysis after blotting the gel to a PVDF membrane. The amino acid sequence ASGGG was observed by N-terminal Edman degradation from the band with a slower mobility, indicating that the N-terminal Met and Cys have been lost (labelled *lin* in Fig. 4a). On the other hand, the sequence of the N-terminus of the faster migrating band could not be determined, indicating that the N-terminus of this protein is protected (indicated by *circ* in Fig. 4a). This is also consistent with the fact that the cyclization can be prevented by eluting the protein into denaturants or by elution with nitrogen nucleophiles such as NH_2OH, both exclusively resulting in the upper band in SDS-PAGE (data not shown). Second, after removing the linear form by heat incubation (see below), the molecular mass of the presumed circular form of BLA was measured by ESI-mass spectroscopy, resulting in a mass about 16 ± 2 Da smaller than the expected molecular mass of the linear form (Fig. 5). Third, the re-linearization of the circular protein by limited proteolysis was tested. For this purpose, a variant of BLA, BLA-Trb, was constructed with the sequence LVPRG introduced at the end of BLA, such that the successful cyclization would yield the sequence LVPRGC, which is similar to that of the thrombin-specific recognition sequence LVPRGS [29] (Fig. 3). Indeed, the digestion of cyclic BLA-Trb by thrombin resulted in a linear form of BLA-Trb, which migrates more slowly than the circular form and co-migrates exactly with the linear form (Fig. 6). The disappearance of the lower band (circular form) and the re-appearance of the upper band (linear form) after thrombin digestion thus indicates that circular BLA-Trb could be re-linearized into the linear form by limited proteolysis. A minor form of about 60 kDa was also seen, which was digested by thrombin. This form is most likely derived from an inter-molecular ligation reaction (see below).

3.1. Stability against exoproteolytic digestion

Since circular proteins lack both N- and C-termini, they should be insensitive to the proteolysis catalyzed by exopeptidases. Hence, we tested the sensitivity against carboxypeptidase Y. At the C-terminus, a very short tag of three histidine residues was introduced (Fig. 3). Both the cyclized and the linear BLA, carrying this his-tag, can bind to an immobilized metal ion affinity chromatography (IMAC) column. The mix-

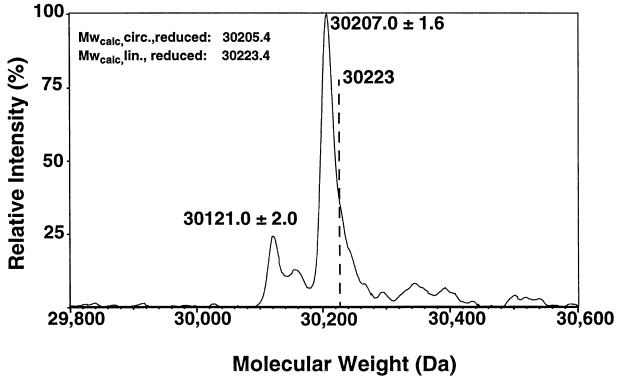


Fig. 5. Mass spectra of BLA. The molecular mass of the circular form of BLA is expected to be 30 205.4 Da. The peak at 30 121 Da corresponds to the linear form which has lost the N-terminal Cys.

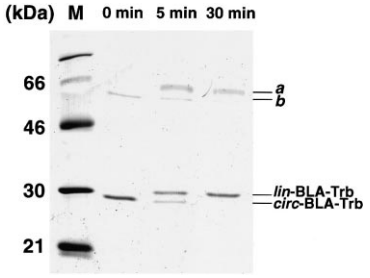


Fig. 6. Time course of the digestion of the circular form of BLA-Trb. 0 min: before addition of thrombin. 5 min, 30 min: time after addition of thrombin. The positions of the circular and linear form of BLA-Trb are indicated by lines. For bands *a* and *b*, see text.

ture of both forms obtained from the cyclization reaction was then treated with carboxypeptidase Y and passed through the IMAC column. In the presence of 10 mM imidazole, to suppress any non-specific binding, the linear form passed right through the column without binding, as the his-tag had been quantitatively removed by the exopeptidase (Fig. 7b, lane 2 and 3). The elution fraction from the IMAC column only contained the lower band, which is the circular form retaining the his-tag (Fig. 7b, lane 4). This experiment clearly demonstrates the resistance of the circular form to proteolysis by carboxypeptidase as well as constituting an efficient method to purify the circular protein from the linear form.

3.2. Stability enhancement by backbone cyclization

The cross-linking of polymer chains will reduce the conformational entropy of their unfolded state, resulting in stabilization of the native state [3]. A N- to C-terminal cyclization of a protein should therefore have the maximal effect on the conformational entropy. Here, we have successfully produced a circular protein of 278 amino acids from a bacterial expression system and could thus test the hypothesis of stabilization by cyclization of a protein. As a first example, we monitored the irreversible aggregation upon heating, because the free energies of related proteins are often well correlated with the temperature at which aggregation initiates, even though there are exceptions [26,27]. Thus, the mixture of linear and circular forms from the reaction mixture was incubated at various temperatures for 1 h. Then, the precipitate and the soluble fraction were quantified by SDS-PAGE as shown in Fig. 4a. The protein bands were analyzed densitometrically and this is plotted in Fig. 4b. At 50°C, nearly 100% of the linear form (indicated as *lin* in Fig. 4a) precipitates, but 50% of the circular form (indicated by *circ* in Fig. 4a) stays in the soluble fraction. As the midpoint of the transition (T_m), we obtained 45°C for the linear form of BLA-S and 50°C for the circular form of BLA-S under these conditions, indicating a stability enhancement of 5°C by cyclization. This increased heat stability of the circular form can also be used for the separation of the circular form from the linear one. This purification method was applied in the re-linearization experiment of circular BLA-Trb in Fig. 6.

We observed not only two major bands but also additional weaker bands with larger molecular weights (Figs. 6 and 4a). This can be interpreted by the fact that not only cyclization but also dimerization and polymerization of the protein can take place by an inter-molecular reaction with the activated C-terminal thioester, depending on the protein concentration [13] (Fig. 1a,e). In Fig. 4, there are four predominant bands in the reaction mixture (indicated by *circ*, *lin*, *a* and *b*). We have identified the lower two bands (*circ* and *lin* in Fig. 4) of about 30 kDa as the circular and linear monomeric forms. One of the additional bands of about 60 kDa, most likely the upper one, is expected to be the linear dimer (labelled *a* in Fig. 4). Such a polymerization of proteins has been observed in other proteins [13]. The nature of the lower other band of about 60 kDa (labelled *b* in Fig. 4), which is more stable against heat precipitation, is not clear and the N-terminus of that protein was not accessible by N-terminal Edman degradation. Further work is required to delineate the topology of these dimeric species.

4. Discussion

The utility of backbone cyclization has been well appreciated in peptides and it has been demonstrated for a large number of peptidic systems that their biological activity and in vivo stability can be improved by cyclization. Therefore, cyclization of small peptides has often been used for restricting their conformation to the active form for pharmaceutical applications (see [8] for a review). The only circular protein,

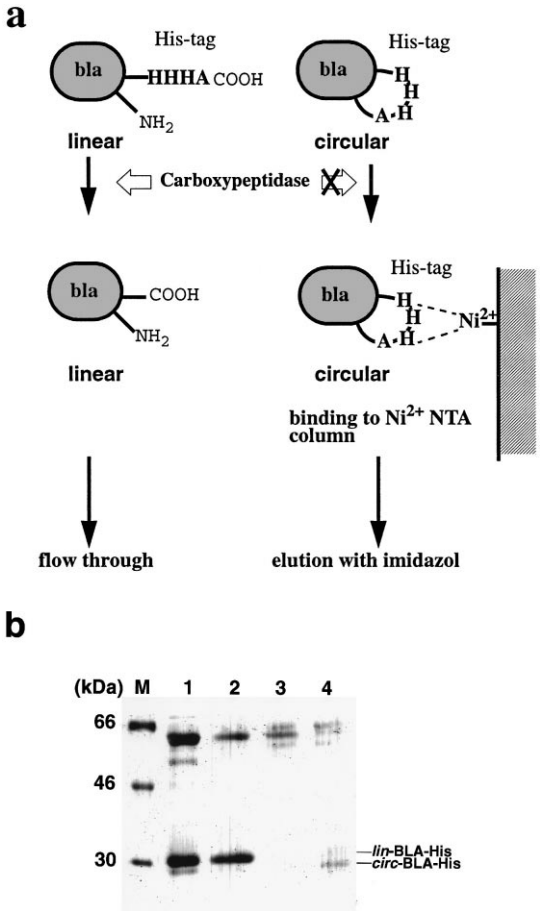


Fig. 7. (a) Schematic drawing of the separation of the circular form from the linear form with a C-terminal his-tag and carboxypeptidase. (b) SDS-PAGE analysis of the purification of the circular form of BLA-His. M: Marker. 1: Before loading on the IMAC column. 2: Flow through from the IMAC column. 3: Wash from the IMAC column. 4: Elution of the IMAC column with 200 mM imidazole.

where the effect on stabilization has been studied, is BPTI [11,10]. However, it contains three disulfide bonds in 58 amino acids and does not fold in the reduced state. Therefore, the comparison had to be made indirectly by investigating the redox reaction of each disulfide bond, making it more difficult to treat the effect of the backbone cyclization quantitatively. No significant stabilization effect by the cyclization of BPTI was observed, possibly because the direct cross-linking of the original N- and C-termini with a peptide bond is structurally not ideal, as it may introduce an unfavorable strain in the native structure [10–12]. Thus, these pioneering experiments may not allow for a general conclusion about the effect of cyclization on stability.

In the present article, we have applied IPL to produce a circular backbone of BLA of 30 kDa. We could indeed demonstrate that the circular form was more stable than the linear form against irreversible aggregation upon heating. Under the conditions of our experiment, no disulfide bond is formed, as the protein was produced in the cytoplasm and kept under reducing conditions. The stabilizing effect of the polypeptide backbone cyclization is consistent with an effect on the conformational entropy of the unfolded state, as predicted by the polymer theory. It also suggests, however, that the aggregation-prone partially unfolded state of the protein, induced by heat incubation, can also be depopulated by cyclization. In contrast to engineering new disulfide bonds, the N- and C-termini, when connected by a 'loose-fitting' loop, will cause essentially no strain on the rest of the structure and this effect should be largely entropic. This is not the case for engineered disulfide bonds [5].

Interestingly, a surprisingly high fraction of proteins appears to have N- and C-termini close to each other [30]. N- or C-termini of proteins are often found to be flexible, as evidenced by NMR or the higher B-factors in X-ray crystallography, where they may even be completely invisible. In these cases, therefore, the cross-linking of the protein termini in close proximity would not be expected to cause any unfavorable constraints on the folded structures. Based on our observation of the stabilizing effect by cyclization, we propose the cross-linking of C- and N-termini in close proximity with a peptide loop as an alternative strategy for protein stabilization and we suggest that this approach might be of rather general utility. The further biophysical characterization of the thermodynamic parameters, as well as the folding and unfolding pathways of both circular and linear forms, will provide more detailed insights into the mechanism of the stability enhancement by the cyclization and the importance of termini in protein folding.

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