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The 5-55 single-disulphide intermediate in folding of bovine pancreatic trypsin inhibitor

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An analogue of the BPTI folding intermediate that contains only the disulphide bond between Cys-5 and Cys-55 has been prepared by mutation of the other four Cys residues to Ser. On the basis of its circular dichroism and ¹H-nuclear magnetic resonance spectra and its electrophoretic mobility, this intermediate is shown to be at least partially folded at low temperatures. This probably accounts for several of the unique properties of this intermediate observed during folding.

Protein folding; Disulphide intermediate; Bovine pancreatic trypsin inhibitor; Nuclear magnetic resonance; Circular dichroism

1. INTRODUCTION

The best-characterized folding pathway of a single-domain protein is that of bovine pancreatic trypsin inhibitor (BPTI), which was elucidated using the relative tendencies of the six Cys residues to form disulphides [1]. The various intermediates with different numbers and identities of disulphide bonds that accumulate transiently during unfolding and refolding can be trapped by carboxymethylation, identified, and characterized. Understanding this folding pathway requires further information about the conformational properties of the intermediates, but detailed studies of the trapped species are complicated by the presence of carboxymethyl groups on the Cys thiol groups not involved in disulphide bonds [2]. To avoid this problem, analogues of the BPTI folding intermediates in which the free Cys residues are mutated to Ser are being subjected to structure analysis.

The single-disulphide intermediate (5-55), with a native-like disulphide bond, has certain unique characteristics during folding of normal BPTI. It does not participate in the rapid equilibrium that occurs dur-

ing folding between the other one-disulphide intermediates, interchange being incomplete after 5 s [3]. It forms a second disulphide bond, 14-38, substantially faster than do the other one-disulphide intermediates [4-6], to produce a non-productive species, (5-55, 14-38). This rapid rate of disulphide formation suggested that (5-55) might adopt a folded conformation that brings Cys-14 and Cys-38 into proximity, as in the native-like conformation of intermediate (30-51, 5-55) [4]. Staley and Kim [7] demonstrated that a peptide model of (5-55), consisting of residues 1 to 9 linked by 6 Gly residues to residues 20 to 33 and by the 5-55 disulphide bond to residues 42 to 58, adopts a metastable conformation that may be native-like. Intermediate (5-55) does not accumulate to substantial levels during folding, probably comprising only 3% of the normal spectrum of one-disulphide intermediates [4]. This low abundance has made it impossible to characterise the trapped (5-55).

The complete analogue of (5-55) has now been prepared by protein engineering and is shown here to adopt a conformation that is at least partially folded at low temperatures.

2. MATERIALS AND METHODS

2.1. Protein expression and purification

The BPTI gene was obtained from a genomic library [8] and manipulated by standard methods to produce a template containing appropriate initiation and termination codons and the desired Cys to Ser mutations. BPTI and its mutant forms were expressed from the pLcHCLM vector [9] as fusion proteins and were released by cyanogen bromide cleavage after a Met residue was introduced at position -1. Purification and disulphide bond formation will be described in detail elsewhere. The final proteins were >90% pure as judged by native gel electrophoresis [10] and by HPLC on two different columns, and they gave satisfactory amino acid analysis.

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; N-BPTI, BPTI with the 5-55, 14-38, and 30-51 disulphide bonds. Unless otherwise stated, Met-52 has been mutated to Arg. (5-55), BPTI with only a single disulphide bond between Cys-5 and -55; other disulphide-bonded intermediates are designated similarly. Unless otherwise stated, Cys residues not involved in disulphide bonds have been mutated to Ser. R-BPTI, BPTI with no disulphide bonds; CD, circular dichroism; NMR, nuclear magnetic resonance; θ , mean residue ellipticity

2.2. CD spectroscopy

CD spectra were recorded on a Jobin-Yvon CD6 dichrograph at protein concentrations of 25 μ M [11] in 10 mM sodium phosphate at pH 7. Cells of pathlength 1 mm and 1 cm were used in the far and near UV ranges, respectively. Spectra were recorded by ten scans at 0.5 nm intervals with a time constant of 2 s, followed by subtraction of the appropriate baseline.

2.3. NMR spectroscopy

1 H NMR spectra were acquired on a Bruker AMX300 spectrometer operating at 300 MHz. The spectra were recorded at 3°C in H₂O at pH 4.6, with 256 scans (plus two dummy scans) of 4096 data points. Specific irradiation of the water resonance occurred for 1.5 s before the start of the pulse and acquisition period. No window functions were applied to the raw data, and no zero filling was performed.

3. RESULTS

Virtually any form of BPTI may be produced with the present expression system, as folding to a stable conformation is not necessary. An insoluble fusion protein is produced, and the cleaved protein is purified in the unfolded, reduced form. The protein cannot, however, include Met residues, because cyanogen bromide is used to cleave the fusion protein. This necessitated replacing the Met residue normally found at position 52 of BPTI; Arg was chosen because it is the most common residue at this position in the natural homologues of BPTI [12]. This mutation produces no change detectable by NMR in the conformation of the protein with three disulphide bonds (unpublished observations). All forms of BPTI produced with this system, including (5-55), retain this mutation.

The relative mobilities in polyacrylamide gels of the various disulphide species reflect primarily the compactness of their hydrodynamic volumes, when there are no differences in numbers of charged groups [13]. Intermediate (5-55) had 87% of the mobility of N-BPTI at 4°C (Fig. 1). Its mobility was much greater than that of R-BPTI in which all the Cys residues had been mutated to Ser residues, and somewhat greater than that of (30-51), the predominant one-disulphide intermediate.

The circular dichroism spectrum of (5-55) at 7°C (Fig. 2) was intermediate between those of R-BPTI and of N-BPTI, which is indistinguishable from that of normal BPTI [11]. The far-UV spectrum of (5-55) showed the shoulders at 216 and 225 nm that are present in the spectrum of N-BPTI but not of R-BPTI. The minimum was red-shifted compared to R-BPTI, a characteristic also shared by N-BPTI and a number of analogues of other partially-folded BPTI folding intermediates. The near-UV spectrum of (5-55) contained all the fine structural details of N-BPTI, albeit at lower intensity. Upon raising the temperature, the CD spectrum of (5-55) shifted toward that of R-BPTI, with a midpoint at approximately 15°C. At 25°C, (5-55) appeared from its CD spectrum to be almost entirely unfolded.

The one-dimensional 1 H NMR spectrum of (5-55) at 3°C included many resonances typical of a folded pro-

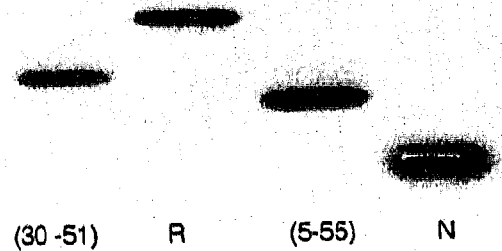


Fig. 1. Electrophoresis of R-BPTI, (30-51), (5-55) and N-BPTI at 4°C in 15% polyacrylamide gels [10].

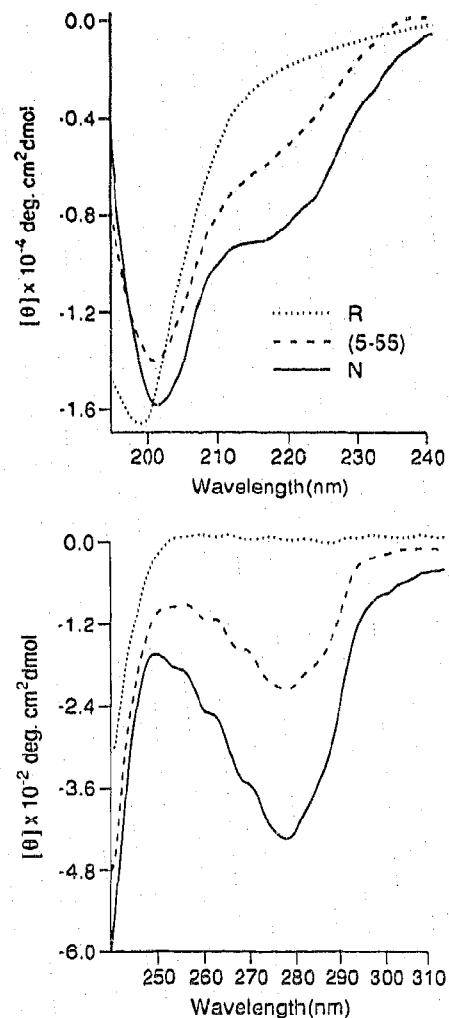


Fig. 2. Circular dichroism spectra of R-BPTI, (5-55) and N-BPTI in the far-UV (A) and near-UV (B) regions measured at 7°C.

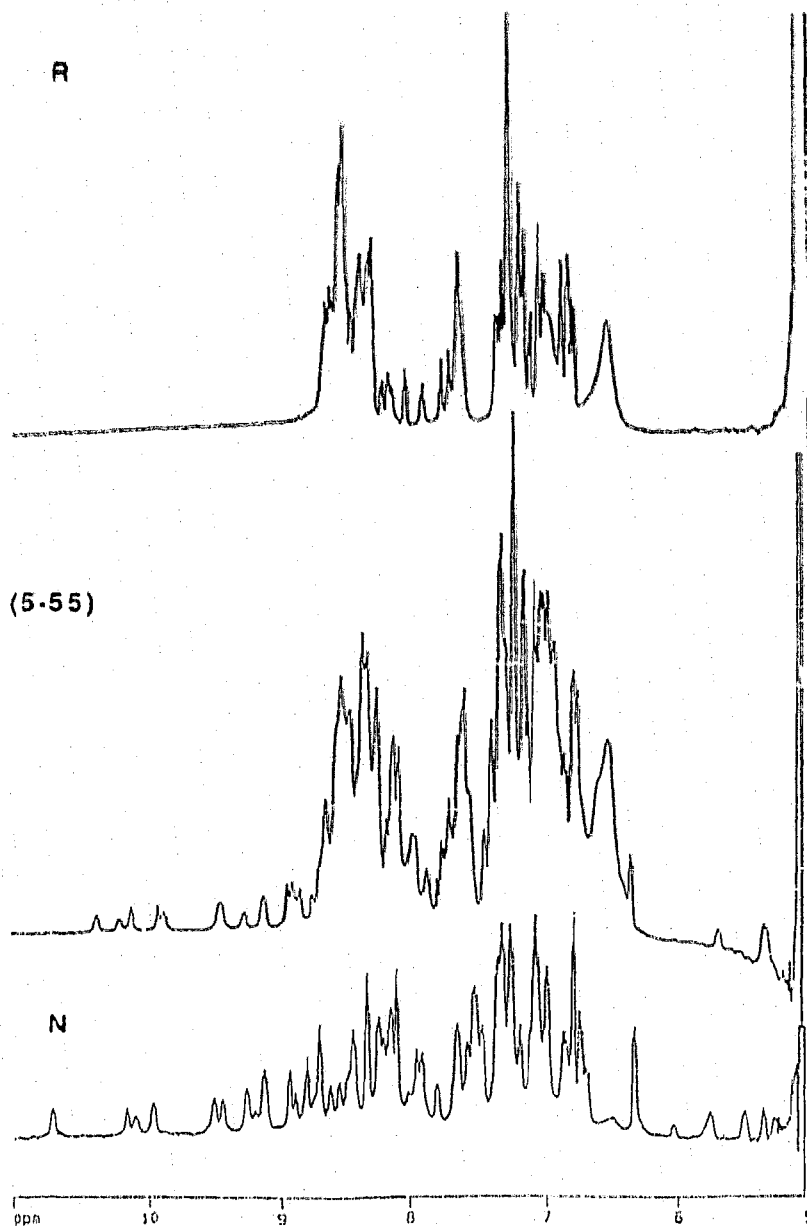


Fig. 3. Aromatic and amide regions of the 500 MHz ^1H NMR spectra of R-BPTI, (5-55), and N-BPTI at 3°C in H_2O .

tein (Fig. 3). Several resonances are dispersed nearly as much as in N-BPTI and much more so than in R-BPTI.

4. DISCUSSION

The one-disulphide intermediate (5-55) in BPTI folding has a relatively compact conformation that is at least partially folded at low temperatures. Its far-UV CD spectrum indicates that the polypeptide backbone has adopted a relatively fixed conformation. Unfortunately, the far-UV CD spectrum of N-BPTI cannot be interpreted in terms of secondary and tertiary structure [11,14]. The near-UV spectrum of (5-55) suggests

that at least some of the aromatic side chains have been immobilized by structural constraints. The near-UV spectrum of N-BPTI is especially intense, probably due to the tight packing of aromatic side chains and disulphide bonds within the interior of this particularly stable small protein [11]. The ^1H NMR spectrum indicated that many hydrogen atoms of the protein exist in unique environments. Further characterization of the conformational properties of (5-55) using two-dimensional NMR analysis is in progress.

The folded conformation present in (5-55) is not very stable, melting out in the range of 10–15°C. Under the usual conditions of folding, at 25°C, the in-

intermediate is folded only a small fraction of the time. Nevertheless, the presence of this conformation during folding probably accounts for the relatively slow rate of formation and reduction of its disulphide bond [5,6] and for its slower rearrangement to the other one-disulphide intermediates [3]. The presence of this conformation during folding is probably also sufficient for the 14-38 disulphide to be formed 50 times faster than other second disulphides in the other intermediates. Yet that rate is only 10% of the rate when the native conformation is fully stable, as in intermediate (30-51, 5-55) [4].

The folded conformation of (5-55) appears to be more ordered than that of the predominant (30-51) intermediate of folding (unpublished data), yet intermediate (5-55) accumulates to lower levels than (30-51) during folding of BPTI. This presumably reflects the greater entropic loss involved in generating interactions between groups at both ends of the polypeptide chain in (5-55). In contrast, intermediate (30-51) may be stabilized by interactions involving primarily the C-terminal half of the polypeptide chain [2,15]. As would be expected, the (5-55) intermediate accumulated to substantially greater levels when the loss in entropy was diminished by linking the chain termini with a peptide bond [16]. The 5-55 disulphide contributes most to stability of the native conformation of BPTI [4], but forming that disulphide is correspondingly more difficult.

In contrast to (5-55), the CD and NMR spectra of R-BPTI with no disulphides give no indication of it adopting a nonrandom conformation, even at low temperatures. Folded conformations are adopted by BPTI only upon formation of the appropriate disulphide bonds [17]. Such conformations do not pre-exist to significant extents in the protein without disulphides (cf. [18]).

A major consideration in interpreting results on the conformational properties of the BPTI disulphide folding intermediates is the state of the Cys residues not involved in disulphide bonds. Their normal thiol groups must be blocked or removed in some way for the intermediates to be trapped and stable. During folding, which has been studied primarily at pH 8.7, the Cys thiol groups are ionized approximately half the time. They are also observed to be exposed to solvent and reactive to thiol and disulphide reagents in all intermediates, except for the quasi-native (5-55, 14-38) [19]. In all other cases, the restrictions on forming disulphides during folding cannot be attributed to the thiol groups being buried in the intermediates [20]. For these reasons, the Cys residues not involved in

disulphide bonds have been replaced by Ser residues here. The stable intermediates then differ from those during folding only in having oxygen atoms in place of sulphur at certain residues. The polar hydroxyls of the Ser residues will undoubtedly tend to remain exposed to water, unless a hydrogen bonding partner happens to be available, but probably not much more than will a partially-ionized thiol group at pH 8.7. Replacing Cys residues with Ala [7,15] will remove this tendency for the reduced protein to remain unfolded. It seems inappropriate to conclude that Cys thiol groups will be buried and unreactive because a replacement nonpolar Ala side chain interacts with the rest of the protein [7,15], especially when the thiol groups that are normally present are known to be reactive during folding [20].

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