A THREE-DISULPHIDE INTERMEDIATE IN REFOLDING OF REDUCED RIBONUCLEASE A WITH A FOLDED CONFORMATION

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1. Introduction

The interaction between cysteine residues of an unfolded protein to form disulphide bonds under appropriate conditions is the most useful current experimental probe of the large conformational transitions which take place during folding to the stable, folded conformation, as it may be controlled experimentally, and the normally unstable and transient intermediates may be trapped in a stable form using the disulphide bonds [1]. This approach has produced the first detailed folding pathway of a globular protein, that of BPTI [2]. A similar study of the larger protein, RNase, the classic subject of protein folding [3–7], extended the earlier studies and trapped and isolated the large number of intermediates that accumulate transiently [8,9]. The 1-disulphide intermediates were found to be essentially a random collection of the 28 possible species, and there were too many 2-, 3- and 4-disulphide intermediates for any of the cysteine residues paired in disulphide bonds to be identified, although these intermediates were not a full random collection. Virtually all of the trapped intermediates had no significant amount of stable, folded conformation on the basis of their spectral and hydrodynamic properties, binding to antibodies against unfolded, reduced RNase, but not to antibodies against native RNase, the absence of an unfolding transition induced by urea, and the absence of enzymic activity [9,10].

The sole exception was a species isolated with the incorrectly refolded, 4-disulphide intermediates, which had substantial enzymic activity and the spectral, hydrodynamic and immunochemical properties of native RNase, but its folded conformation was unfolded by only half the concentration of urea required to unfold native RNase. This species has now been isolated in homogeneous form and will be shown to contain only 3 of the 4 disulphide bonds of native RNase, and thus is designated here as IIIn, and to lack the disulphide between Cys-40 and -95.

Abbreviations: RNase, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor (Kunitz)

2. Materials and methods

The folding intermediates of RNase were trapped by addition of iodoacetate to 0.1 M to a solution of initially reduced RNase (30 μM) which had been refolded for 20 min at 25°C with 0.20 mM oxidized glutathione as disulphide reagent in 0.10 M Tris–HCl buffer (pH 8.7), 0.20 M KCl and 1 mM EDTA [8,9]. After 2 min, the protein was separated from the reagents by gel filtration in 0.1 M acetic acid and was recovered by lyophilization. The intermediates were separated by chromatography on CM-cellulose; the initial half of the peak of 4-disulphide intermediates, designated IV in [9], was subjected to gel filtration to isolate the folded intermediate (see fig.1).

For digestion with thermolysis, 3 mg appropriate protein was dissolved in 0.8 ml 0.25 M ammonium acetate buffer (pH 7.5) also containing 0.25 mM $CaCl_2$ and 0.8 mg thermolysin (Calbiochem). Digestion was at 65°C for 4.5 h; the mixture was then lyophilized.

Diagonal maps of the thermolysin digests were prepared by the procedure in [11] at pH 3.5 exactly as in [12,13]. The peptide spots were detected by dipping the thoroughly dried paper through acetone containing, first, 1% triethylamine, and secondly,

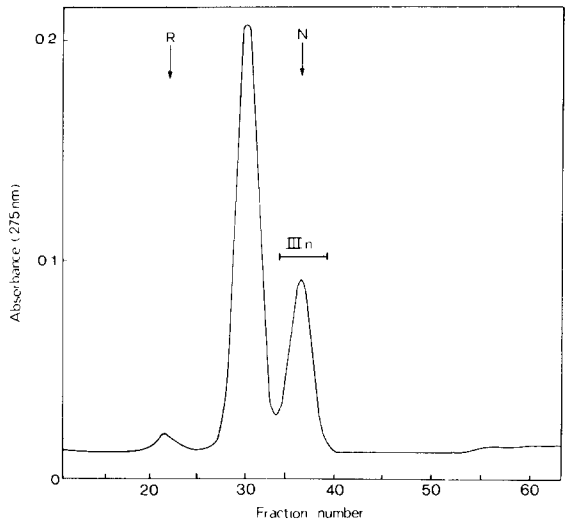


Fig.1. Separation by gel filtration of compact intermediate IIIn from the other, incorrectly-folded 4 disulphide intermediates trapped during refolding of reduced RNase. The trapped intermediates were isolated as the first half of peak IV obtained by chromatography on CM-cellulose [9], concentrated to 3 ml by lyophilization, and then subjected to gel filtration on a column of Biogel P60 (100–200 mesh), 2.5 cm diam. and 80 cm length, in 0.1 M ammonium bicarbonate at room temperature; 5 ml fractions were collected, and the protein was recovered by lyophilization. The elution positions are indicated of fully reduced, carboxymethylated (R) and of native (N) RNase, determined in separate experiments on the same column.

10^{-3}% Fluram (Roche); this procedure can detect peptides occurring in quantities <1% those of the major peptides characterized here [14]. After washing the paper with acetone, all fluorescent spots off the main diagonal were eluted with 0.1 M aqueous NH_3, dried, hydrolyzed for 24 h at 105°C in 6 M HCl containing 1 mM phenol, and subjected to amino acid hydrolysis.

3. Results

3.1. *Isolation of the folded intermediate IIIn*

The compact trapped intermediate IIIn of RNase was found upon CM-cellulose chromatography to be eluted in the first half of the peak of incorrectly-folded 4-disulphide intermediates, designated IV in [9], so this material was further fractionated by gel filtration (fig.1). Intermediate IIIn was well resolved from the other intermediates on the basis of its compact folded nature, eluting in the same position as native RNase and considerably after trapped, reduced RNase and the other intermediates [10]. It must be both compact in conformation and monomeric.

3.2. *The number of disulphides in intermediate IIIn*

Intermediate IIIn was found to contain av. 2.1 carboxymethyl groups introduced by ^{14}C-labelled iodoacetate (6.7 Ci/mol) used to trap the intermediates. When the disulphide bonds of the isolated protein were reduced and the resulting free thiol groups reacted with the uncharged reagent iodoacetamide, varying mixtures of iodoacetamide and the acidic reagent iodoacetate, and with just iodoacetate, 6 additional bands were observed electrophoretically to have been generated by the varying extents of reaction with the acidic reagent, indicating the presence of 6 free cysteine residues [15,16]. This procedure has been shown to demonstrate the integral number of free cysteine residues/polypeptide chain and to give the correct value of 8 with normal RNase [16].

Therefore, intermediate IIIn has 3 intramolecular disulphide bonds, and the 2 other Cys residues were carboxymethylated upon trapping.

3.3. *Identification of the Cys residues paired in disulphide bonds and those carboxymethylated*

The identity of intermediate IIIn was established by diagonal electrophoresis [11] at pH 3.5 of thermolysin digests, as this procedure has been shown with BPTI to identify the pairs of Cys residues involved in disulphide bonds and those carboxymethylated [12,13]. The diagonal map of native RNase (fig.3a) and the identification of the major peptides of the primary structure [17] by their amino acid compositions (table 1), indicated unambiguously the 4 disulphide bonds linking Cys 26–84, 40–95, 58–110 and 65–72 [18,19]. All the significant peptides were identified, and there were no indications of disulphide interchange.

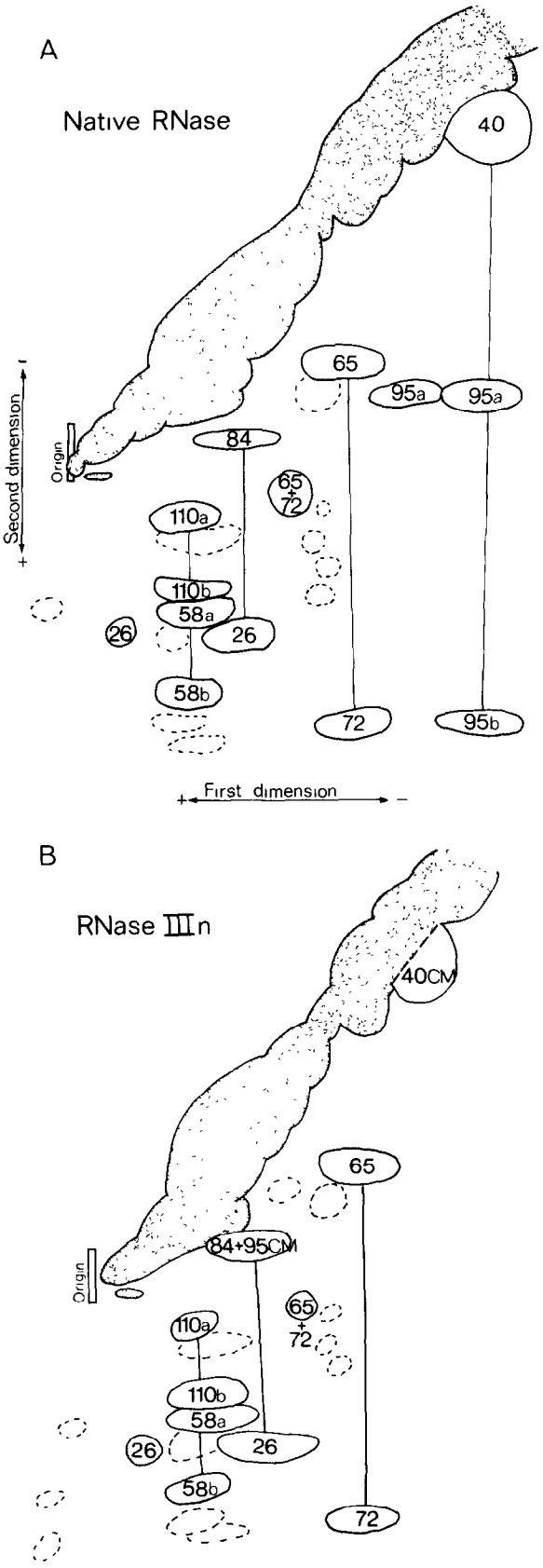
Intermediate IIIn gave a very similar diagonal map but for the conspicuous absence of the peptide spots resulting from disulphide 40–95 (fig.2b). The corresponding peptides were found, but they had different mobilities in the first dimension, indicating that they were not linked by a disulphide bond, and their positions just below the diagonal, plus their amino acid compositions (table 1), indicated that the Cys residue of each was carboxymethylated; such peptides are somewhat more acidic at pH 3.5 in the second

Table 1
Identification of the cysteine-containing peptides from their amino acid compositions

Peptide residues	110a 108–114	58a 57–59	58b 57–58	26 25–26	84 + 95 CM 88–95[a] 81–87	65 + 72 63–72	65 63–68	72 71–72	40 CM 35–42	84 81–87[b]	95a 88–95[b]	95b 94–95[b]	40 35–42[b]
$CysO_3H$	1.18 (1)	1.15 (1)	1.16 (1)	1.10 (1)	0.81 (0.5)[c]	2.03 (2)	1.08 (1)	1.02 (1)	0.41[c]	1.08 (1)	1.22 (1)	1.09 (1)	1.14 (1)
Asp	1.06 (1)		0.29 (0)		1.79 (1.5)	1.97 (2)	1.02 (1)	*0.98* (1)	1.16 (1)	1.17 (1)	1.26 (1)	*0.91* (1)	1.12 (1)
$MetO_2$													
Thr					0.86 (1.0)	0.99 (1)			1.11 (1)	1.58 (2)			1.02 (1)
Ser	0.52 (0)	0.94 (1)	0.30 (0)		1.66 (2.0)				0.38 (0)	0.31 (0)	1.78 (2)		
Glu	0.93 (1)				0.61 (0.5)	1.11 (1)				1.19 (1)			
Pro	0.67 (1)				1.15 (1.0)				0.77 (1)		1.05 (1)		0.99 (1)
Gly	0.99 (1)				*0.57* (1.0)	1.10 (1)	0.92 (1)		0.30 (0)	0.37 (0)	*0.80* (1)		
Ala	1.13 (1)					1.05 (1)	1.09 (1)						
Val	*1.03* (1)	*0.91* (1)	*0.84* (1)			*0.92* (1)	*0.98* (1)						
Ile	0.37 (0)				*0.53* (0.5)					*0.97* (1)			
Leu									*0.80* (1)				*0.81* (1)
Tyr				*0.90* (1)	1.08 (1.0)						1.04 (1)		
Phe													
His													
Lys					1.00 (1.0)	0.83 (1)	0.90 (1)		2.05 (2)		0.84 (1)		1.99 (2)
Arg					0.43 (0.5)				1.08 (1)	1.01 (1)			0.94 (1)

[a] A mixture containing ~2 mol peptide 88–95 CM to 1 mol 81–87 is indicated by the amino acid composition and assumed for the expected molar values
[b] Peptides from diagonal map of native RNase
[c] Carboxymethyl cysteine sulphone is largely destroyed during acid hydrolysis

The observed molar ratios of the peptides eluted directly from the diagonal maps of fig.2 are presented, followed by the expected number in parentheses. The amino-terminal residue is italicized as it is generally recovered in rather low yields, presumably due to reaction with the fluorescamine. The peptides are those from the diagonal map of intermediate IIIn, except where indicated for those peptides unique to native RNase, which were from that diagonal map. All amino acids present with a molar ratio >0.2 are reported



dimension due to performic acid oxidation of the carboxymethyl cysteine residue to the sulphone [12,13]. The peptides resulting from the 3 disulphides 26–84, 58–110 and 65–72 were identified and confirmed to be the same as those observed with native RNase (table 1).

4. Discussion

RNase intermediate IIIn with the 3 native-like disulphides linking Cys residues 26–84, 58–110 and 65–72, but with Cys-40 and -95 carboxymethylated after trapping, has a compact native-like conformation on the basis of its mobility through polyacrylamide gels and upon gel filtration, its reaction with antibodies against native RNase, its enzymic activity, and its absorbance, circular dichroism, and Raman spectra [9,10]. It has the same electrophoretic mobility as native RNase at pH 3.8, but appears by ion-

Fig.2. Determination of the disulphide bonds of intermediate IIIn by diagonal electrophoresis. Thermolysin digests of native RNase (A) and intermediate IIIn (B) were subjected to electrophoresis at pH 3.5 in the horizontal direction; peptides linked by disulphide bonds migrate together. The paper was then subjected to performic acid-oxidation to cleave the disulphides, converting the cysteine residues to cysteic acid; carboxymethyl cysteine residues are oxidized to the sulphone. The electrophoresis was then repeated in the vertical direction. Peptides originally linked by disulphides are more acidic and are paired by their common mobility in the first dimension.

All the peptides stained by fluorescamine were eluted and identified by their amino acid composition (table 1); the major peptides are designated by the residue number of the cysteine residue they contain. Peptides originally linked by disulphides are joined by vertical lines; 'CM' indicates that the cysteine residue was carboxymethylated. The diagonal of peptides with the same mobility in both directions is shaded.

Two spots containing peptide 25–26 were found; that to the left and unpaired, was present in much lower amounts, but the peptide to which it was originally linked was not found; presumably it was a larger peptide including Cys-84 and remained near the diagonal. Similarly, a second, unpaired spot containing peptide 88–95 was observed, probably due to similar circumstances. In addition to peptides 63–68 and 71–78, which were originally linked by disulphide 65–72, the uncleaved peptide of 63–72 containing the intramolecular disulphide was also present. Peptide 110b was found by electrophoresis at pH 2.1 to consist primarily of residues 108–110, but it was lost during quantitative amino acid analysis.

exchange chromatography to be more acidic at pH 6.2, so the p*K* values of the 2 carboxymethyl groups appear to be somewhere between these 2 pH values.

In native RNase, the 40–95 disulphide bond is located at one extremity of the folded conformation, not far from the surface of the molecule, but far from the active site (fig.3), so it is perhaps not surprising that it has a stable conformation and is enzymically active. The 40–95 disulphide of native RNase is that most accessible to the solvent [20,21], although it is well-defined crystallographically [22], perhaps indicating a relatively rigid conformation around it.

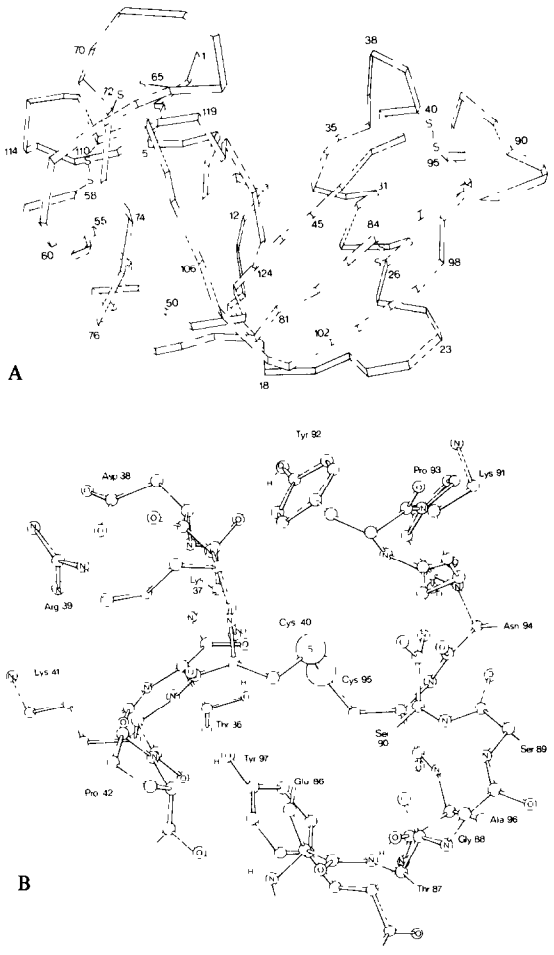


Fig.3. The native conformation of RNase, showing the positions of the 4 disulphide bonds and the environment of the 40–95 disulphide bond. The ribbon diagram in (A) connecting the α carbon atoms was drawn from fig.7.7 of [34]. The local environment of the 40–95 disulphide bond is shown in (B) from a similar viewpoint; it was drawn using the coordinates of ribonuclease-S [22].

Nevertheless, its absence in intermediate IIIn causes the folded conformation to be less stable, with the disulphides kept intact, being disrupted by only half the concentration of urea required to unfold native RNase [9]. Assuming the stability to be related linearly to the urea concentration [23], the conformational stability of IIIn in the absence of urea is likely to be only half the 10.6 kcal/mol estimated for native RNase [24]. This decrease in stability of 5 kcal/mol is about that expected simply for the absence of 1 covalent crosslink [25].

The 40–95 disulphide in native RNase is near one of the 2 *cis* peptide bonds, that between Tyr-92 and Pro-93 [19], which is a slightly less favourable conformation than the normal *trans* isomer [26–28]. In the absence of the 40–95 disulphide bond, the conformation of this polypeptide loop may be altered to permit the *trans* form of the peptide bond, contributing slightly to the stability of the folded conformation. The circular dischroism spectra suggest a slight change in conformation in IIIn, particularly of tyrosine residues [10]; the 40–95 disulphide bond is normally sandwiched between Tyr-92 and -97 (fig.3), two of the 'buried' tyrosine residues of normal RNase [19], but the conformational perturbation may simply be due to the carboxymethyl groups.

Intermediate IIIn has been detected only as an intermediate in refolding of reduced RNase. It has not accumulated to detectable levels during unfolding of native RNase upon disulphide reduction, nor have any other intermediates [9]; if IIIn is an intermediate in unfolding, its 3 disulphide bonds must be reduced very rapidly. It is thus not comparable to intermediate (30–51, 5–55) in the BPTI pathway [1,2] which can readily make and break the 14–38 disulphide bond, but is more like intermediate (30–51, 14–38), which is not formed directly upon unfolding and cannot readily form the third disulphide bond.

The direct pathway of refolding of RNase is especially complicated by slow *cis–trans* isomerization of peptide bonds adjacent to the 4 proline residues [26,29,30], due to the presence of 2 *cis* bonds in the native conformation [19,22]. If the independent probability of each such peptide bond being *trans* in the unfolded state is 0.8 ± 0.1, the fraction of unfolded molecules having one or more incorrect isomers will vary from 95.6–99.2%; ~80% of molecules unfolded, but with the 4 disulphides kept intact, refold slowly due to incorrect *cis–trans* iso-

mers [29,31]. It is then possible that intermediate IIIn represents the nearly complete refolding of those molecules of reduced RNase which had a *trans* isomer of the peptide bond adjacent to Pro-93, or a *cis* isomer by Pro-42, with the other bonds correct, making possible refolding to a native-like conformation, but with the conformation in this part of the molecule altered by the incorrect *cis–trans* isomer; the 40–95 disulphide bond then would not be formed before slow isomerization of the peptide bond.

Intermediate IIIn is the only intermediate with a stable, folded conformation that has been detected in this study. It is different from 2 other folded forms of RNase with only 2 and 3 disulphide bonds that have been reported, one lacking the 65–72 disulphide [32], the other lacking both 65–72 and 58–110 [33]; they have not been detected here.

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