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On the relevance of non-random polypeptide conformations for protein folding

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The possibility that any non-random conformation in reduced bovine pancreatic trypsin inhibitor (BPTI) and ribonuclease A might be significant for folding has been considered, using the experimental data available on forming the first disulphide bond in each. It is a thermodynamic necessity that whatever conformation stabilises a particular disulphide bond be stabilised to the same extent by the presence of the disulphide. The stabilising effects of disulphides are known approximately, so the stability of any non-random conformation found in a one-disulphide intermediate can be estimated in the absence of the disulphide bond. The non-random conformation in the BPTI intermediates is sufficiently labile to indicate that it would be expected to be present in no more than 5% of the reduced BPTI molecules. There is much less non-random conformation apparent in ribonuclease A. Whatever conformations are represented in the bulk of these two reduced proteins cannot favour disulphide formation and further productive folding.

1. Introduction

Unfolded proteins have generally been considered to approximate random coils, primarily on the basis of the demonstration by Tanford [1] that they have the appropriate dimensions in strong denaturants. Other properties of unfolded proteins generally are also like those expected of random coils.

Most proteins require concentrated solutions of denaturants to unfold them, but proteins with disulphide bonds are often unfolded simply by reducing more than one of the disulphides. Even in the absence of denaturants, such proteins appear to be close to random coils. For example, reduced bovine pancreatic trypsin inhibitor (BPTI) is similar to a random coil in its hydrodynamic volume [2,3], circular dichroism spectrum [4,5], ^{1}H-NMR spectrum [6], exposure of its aromatic side chains to solvent [7], immunochemical properties [8], reactivity of its thiol groups with external reagents [9], and the effective concentrations of the thiol groups relative to each other [10,11]. Similar data are available for reduced ribonuclease A [12–14].

The random-coil properties of unfolded proteins were not unexpected, in view of the weakness in water of the individual interactions that might be responsible for non-random conformations [15], the general absence of ordered structures in peptides in water [16], the large conformational entropy of a disordered polypeptide [17], and the cooperativity of protein folding transitions [18]. Nevertheless, it is difficult to prove experimentally that any polypeptide chain is a random coil.

There are now many reports that unfolded proteins are not truly random (e.g., refs. 19–22). Upon transfer to conditions favouring folding, several unfolded proteins have been observed directly to collapse, rapidly and reversibly, to com-

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pact but non-native conformations [23,24]. Detailed studies have detected a variety of non-random conformations in small peptides [25–27], and one short α-helix has been found to be more stable in isolation than expected [28,29]. Consequently, considerable uncertainty exists about the exact nature of the unfolded state, and it is possible that it is less random than previously thought.

The question then arises as to the significance of any non-random conformation in unfolded proteins. Such conformations are often envisaged to be used as nucleation sites in protein folding [30] or 'kernels' with which to 'seed' protein folding [31,32]. On the other hand, there is no compelling evidence that this is the case. The purpose of this article is to examine critically the possible role that any such non-random conformation might have for refolding, using the best characterised steps in folding, namely, forming the first disulphide bonds in reduced BPTI and ribonuclease A.

2. Disulphide formation in reduced proteins

2.1. Bovine pancreatic trypsin inhibitor (BPTI)

The first disulphide bond to be formed in reduced BPTI generates a very non-random spectrum of one-disulphide intermediates: about 60% of the molecules have the native-like disulphide bond between Cys 30 and 51, designated intermediate (30–51), about 30% have the non-native disulphide 5–30 [33], while the other 13 possible disulphides make up the remaining 10%. The predominance of intermediate (30–51) is kinetically important for further productive folding, in which the 30–51 disulphide bond is retained.

The one-disulphide intermediates could be envisaged as resulting from a non-random conformation in reduced BPTI that favours proximity of certain Cys residues. However, further analysis demonstrates that any such non-random conformation can be present to only a very small extent in reduced BPTI.

2.1.1. Effects of 8 M urea

The presence of 8 M urea during folding produces an approximately random spectrum of one-disulphide intermediates [34]. That each of the 15 possibilities is present in its expected random proportion has not been demonstrated, but the broad mixture of all possible disulphides, with none predominating, is at least a good approximation. Therefore, 8 M urea drastically destabilises the normal one-disulphide intermediates and is likely to do so also to any non-random conformation in the reduced protein that was responsible for them.

Table 1

Kinetics of forming the first disulphide bonds in reduced BPTI

The reagent was dithiothreitol in its oxidised and reduced forms, because the rate constant for forming the protein disulphide is directly proportional to the rate of the intramolecular step involving the conformational changes in the protein. Conditions were 0.1 M Tris-HCl (pH 8.7), 0.2 M KCl, 1 mM EDTA at 25 °C [11,34].

	[Urea]	
	0	8 M
Rate constants		
Forward (s^{-1} M^{-1})	2.2×10^{-2}	1.6×10^{-2}
Intramolecular (s^{-1})	6.6	4.8
Reverse (s^{-1} M^{-1})	20	49
Equilibrium constant	1.1×10^{-3}	0.33×10^{-3}

The intramolecular rate constant for forming the first disulphide bonds should be decreased by 8 M urea if certain disulphides are favoured by a non-random conformation in the reduced protein. The experimental results listed in table 1 demonstrate that very little effect is observed. Therefore, it is unlikely that, in the reduced protein, pairs of Cys residues are in closer proximity, on average, than in the urea-unfolded protein.

Urea does destabilise the one-disulphide intermediates because the equilibrium constant for their formation is decreased by a factor of 3 (table 1). This is primarily due to an increase in the rate of reduction of the one-disulphide intermediates, and indicates that the effect of urea is primarily on the one-disulphide intermediates, not on the reduced protein.

2.1.2. Microscopic steps in forming the first disulphide bonds

All six Cys thiols of reduced BPTI participate in forming the first disulphide bond, in both the

presence and absence of urea. They have comparable reactivities with thiol reagents, even in the absence of any denaturant, virtually the same as that of model thiols [9], yet none accumulate detectably as mixed disulphides with an intermolecular disulphide reagent like oxidised glutathione [35,36]. Therefore, all six Cys residues can rapidly form an intramolecular disulphide bond with at least one of the other Cys residues. Blocking irreversibly one or two of the Cys residues not involved in the predominant 30–51 disulphide bond substantially decreases the rate of its formation [36], so these Cys residues normally contribute to this rate.

The observed rate of forming the first disulphide bond in reduced BPTI is the sum of the individual reactions generating all the possible one-disulphide intermediates. It is not yet possible to apportion the contribution of each disulphide bond to the total rate. In an ideal random coil with large numbers of residues between the Cys residues, the relative contribution of each pair should vary as $n^{-3/2}$, where $n-1$ is the number of residues between them [37,38]. Such relationships, however, are known not to apply to short segments, where stereochemical constraints can either increase or decrease dramatically the tendencies of pairs of groups to come into proximity [39–41]. The values measured with reduced BPTI have been alleged [42] to be one order of magnitude larger than expected from the sum of the individual values predicted by $n^{-3/2}$ relationship, but this is probably due to the inapplicability of this relationship to reduced BPTI, where the value of n is as small as 4.

The observed values of the intramolecular rate constants for forming the first disulphide bond, in both the absence and presence of urea, are approximately those expected for a disordered polypeptide chain. Comparing the intramolecular rate constant to that for the equivalent intermolecular reaction gives the effective concentration of the pairs of Cys thiols relative to each other; this value may then be compared with those obtained with other polymers and other interactions. Polymers containing 10–20 monomer units between the reactive groups have effective concentrations in the range 0.01–0.08 M [41]. The sum of the effective concentrations of the 15 disulphides possible in reduced BPTI is 0.66 M normally and 0.48 M in the presence of 8 M urea. Therefore, the average value is 0.03–0.04 M, within the range observed for other disordered polymers.

The normal non-random spectrum of BPTI one-disulphide intermediates results because the disulphides initially formed are interchanged intramolecularly and rapidly. This was inferred initially from the identical kinetic behaviour of the various one-disulphide intermediates, even though they have different kinetic roles [43]. It was shown directly by generating the approximately random spectrum in 6 M guanidinium chloride, trapping it with acid, and removing the denaturant by gel filtration; upon jumping the pH to normal, the non-random spectrum of intermediates was generated very rapidly [34]. Therefore, the disulphides formed initially could be very random, even though a non-random spectrum results.

2.1.3. Non-random conformation in the (30–51) intermediate

The relative levels of the one-disulphide intermediates reflect their relative free energies. The most favourable of these, intermediate (30–51), is only 0.4 kcal/mol more stable than the next most predominant, intermediate (5–30), and only approx. 2.6 kcal/mol more stable than each of the other 13 possible intermediates.

The favourable stability of intermediate (30–51) under normal conditions is a result of its favourable conformational properties [6,8,42], and both the stability and non-random conformation of the intermediate are destabilised by urea and by higher temperatures [6,34,44]. Intermediate (30–51) normally exists in a non-random, at least partially folded, conformation that is in equilibrium with an unfolded form [6,8]. The stability of this non-random conformation can be measured experimentally in three different and independent ways that give consistent results:

(1) The conformational equilibrium of the trapped intermediate has been observed directly by NMR at various temperatures [6]. The equilibrium constant between the non-random,

$(30–51)_{NR}$, and the unfolded, $(30–51)_U$, conformations

$$K_{conf} = \frac{[(30–51)_{NR}]}{[(30–51)_U]} \quad (1)$$

was observed directly to have a value of approx. 20 at 25 °C, with a single standard deviation encompassing the range 5–52 [6]. The intermediate studied in this way has carboxymethyl groups blocking the free Cys thiols, which might destabilise the non-random conformation. Therefore, this value might be lower than that applying during folding, when the thiol groups are free, as measured in the following two ways.

(2) The value of K_{conf} during folding can be estimated from the ratio of one-disulphide intermediates during folding, because intermediate $(30–51)_U$ will be in equilibrium with the other one-disulphide intermediates, which are assumed to be largely unfolded. Intermediate (5–30) is omitted because its high level indicates that it must also have a non-random conformation. The equilibrium constant between the unfolded forms should be statistically weighted by the entropic differences of the 15 possible disulphide bonds, but, as discussed above, this is very uncertain for such small numbers of residues in BPTI, so it will be assumed that the 30–51 disulphide bond has an average statistical probability in the unfolded protein of:

$$\frac{[\text{Others}]}{[(30–51)_U]} = 14 \quad (2)$$

During folding, about 60% of the molecules have the 30–51 disulphide, while about 10% have all the others (neglecting the special (5–30)), so

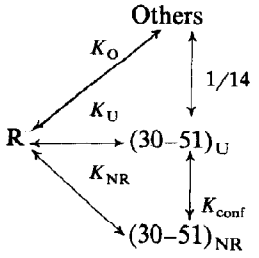
$$\frac{[(30–51)_{NR}] + [(30–51)_U]}{[\text{Others}]} = \frac{60}{10} \quad (3)$$

$$\frac{[(30–51)_U](K_{conf} + 1)}{[\text{Others}]} = 6 \quad (4)$$

$$\frac{(K_{conf} + 1)}{14} = 6 \quad (5)$$

$$K_{conf} = 83 \quad (6)$$

(3) The effects of 8 M urea on the stability of the first disulphide bonds formed in reduced BPTI, R, can be interpreted by assuming that the only effect of the urea was to make negligible the amount of intermediate (30–51) with a non-random conformation:



(7)

The thiol and disulphide reagents involved in breaking and forming the protein disulphides can be neglected here. In the presence of 8 M urea, the observed equilibrium constant for disulphide formation is

$$K_{obs,U} = \frac{[\text{Others}] + [(30–51)_U]}{[R]} \quad (8)$$

$$K_{obs,U} = K_O + K_U = \tfrac{15}{14} K_O \quad (9)$$

In the absence of urea,

$$K_{obs} = \tfrac{15}{14} K_O + K_{NR} = \tfrac{15}{14} K_O + \frac{K_{conf}}{14} K_O \quad (10)$$

$$K_{obs} = \frac{K_O}{14}(15 + K_{conf}) \quad (11)$$

The ratio of the two observed values (table 1) is

$$\frac{K_{obs}}{K_{obs,U}} = \frac{15 + K_{conf}}{15} = \frac{1.1 \times 10^{-3}}{0.33 \times 10^{-3}} \quad (12)$$

$$K_{conf} = 35 \quad (13)$$

The three values estimated by the three independent methods are satisfactorily similar. The somewhat lower value estimated by the first method can be attributed to the presence of the blocking groups on the free Cys thiols. The agreement between the different values is evidence for the validity of the experimental measurements and their interpretation here. It seems clear that the non-random conformation of intermediate (30–51) at 25 °C is normally between 1.8 and 2.6 kcal/mol more stable than the unfolded form with the same

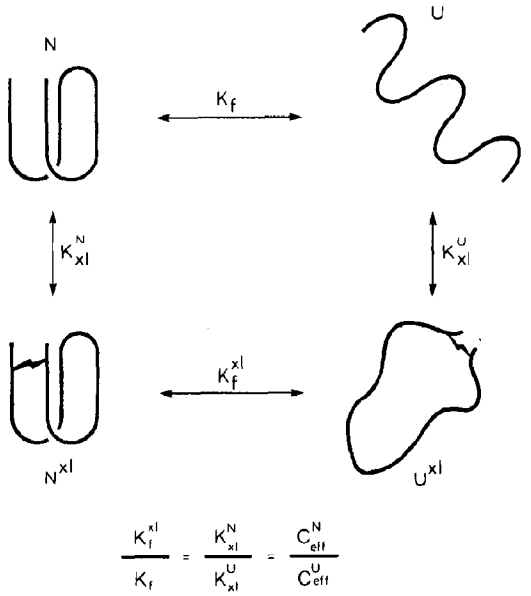


Fig. 1. Relationship between cross-link stability and its contribution to conformational stability. The effect of the cross-link on stability of the folded state, N, must be exactly the same as the effect of the conformation on the stability of the cross-link. The latter is proportional to the ratio of the effective concentrations (C_{eff}) of the cross-linked groups in the folded and unfolded states. Therefore, the relative stabilities of a disulphide bond in the folded and unfolded states should be the same as the effect of the disulphide on the conformational stability of the protein.

disulphide bond, or each of the other unfolded intermediates.

2.1.4. Non-random conformation in the reduced protein

It can now be shown that the marginal stability of this non-random conformation in the presence of the 30–51 disulphide bond implies that it would not be substantially populated in the absence of the disulphide bond, in reduced BPTI.

Disulphide bond stability and conformational stability are linked functions (fig. 1). Therefore, whatever conformation stabilises a particular disulphide bond must be stabilised to exactly the same extent by the presence of the disulphide bond. The stabilising effects of disulphide bonds are usually attributed to their destabilising effect on the unfolded protein, by decreasing its conformational entropy [45–48]. With 20 amino acid residues between Cys 30 and 51, this is expected to be about a factor of approx. 10^2 [46,47]. The stability of the (30–51) non-random conformation in the one-disulphide intermediates should be diminished in the fully reduced protein by this factor.

The appropriate equilibrium to consider is the ratio of molecules in the one-disulphide intermediates having the (30–51) non-random conformation to those that are unfolded. From eqs. 1 and 2 and the various estimates of K_{conf},

$$\frac{[(30\text{–}51)_{NR}]}{[(30\text{–}51)_U] + [\text{Others}]} = \frac{K_{conf}}{15} = 1.3\text{–}5.5 \quad (14)$$

With a 10^2-fold lower ratio, the fully reduced protein would be expected to have this non-random conformation no more than 1–5% of the time.

The classical interpretation of the conformational effect of cross-links has been found to underestimate those observed in folded BPTI, because it assumes that all disulphides have the same stability in the folded state [49,50]. Disulphide bonds within unfolded proteins can be much more stable than was imagined previously [10,11,47], and consequently can impart much more stabilisation. For example, the 14–38 disulphide bond of native BPTI increases the equilibrium constant for the folded conformation by a factor of between 5.3×10^5 and 2.7×10^6 [51], even though it is the weakest disulphide in folded BPTI [10,11,47]. This reflects the very different stabilities of the disulphide bonds in the folded and unfolded states:

$$\begin{array}{ccc} & \xrightarrow{K_{SS}^U} & \\ (30\text{–}51,5\text{–}55)_U & \longleftrightarrow & (30\text{–}51,5\text{–}55,14\text{–}38)_U \\ \Big\updownarrow K_{conf}^{SH} & & \Big\updownarrow K_{conf}^{SS} \\ N(14SH,38SH) & \longleftrightarrow & N \\ & \xrightarrow{K_{SS}^N} & \end{array} \quad (15)$$

where N is folded BPTI with all three disulphides, unless indicated otherwise. The data of Schwarz et al. [51] show that at 25 °C

$$\frac{K_{conf}^{SS}}{K_{conf}^{SH}} = 5.3 \times 10^5\text{–}2.7 \times 10^6 \quad (16)$$

For the unfolding to take place without disulphide interchange, the two thiols had to be blocked irreversibly, and two different blocking groups produced the two slightly different results.

The stability of the 14–38 disulphide bond in folded BPTI is known experimentally, and can be expressed as an effective concentration of the two thiols in folded BPTI of 2.3×10^2 M [11]. The corresponding value in the unfolded protein with two disulphide bonds is not known. The value in the reduced protein is probably about 0.04 M, but this would give a ratio for the folded and unfolded states of only

$$\frac{K_{SS}^{N}}{K_{SS}^{U}} = \frac{2.3 \times 10^2 \text{ M}}{0.04 \text{ M}} = 5.8 \times 10^3 \qquad (17)$$

i.e., some two orders of magnitude smaller than is indicated by the conformational equilibrium values. The latter indicate that the stability of the 14–38 disulphide bond in the unfolded state is lower, with an effective concentration of only 8×10^{-5}–4×10^{-4} M; such values are feasible if the two disulphides present keep the Cys 14 and 38 thiols apart in the unfolded protein. Alternative explanations of strain in the folded state as a result of the presence of the two thiols and their blocking groups seem unlikely because these two Cys side chains are on the surface of the protein and fully exposed to solvent [52]. In any case, these results demonstrate that the analysis of fig. 1 does not overestimate the stabilising effects of disulphides, but rather underestimates them, even though it predicts larger effects than the classical theory.

This new analysis of the initial disulphides in reduced BPTI indicates that non-random conformation in reduced BPTI responsible for the (30–51) intermediate might be even less populated than estimated previously:

$$\begin{array}{ccc} & \xrightarrow{K_{SS}^{U}} & \\ R & \longrightarrow & \text{Others} + (30\text{–}51)_U \\ \Big\uparrow K_{conf}^{R} & & \Big\uparrow K_{conf}^{I} \\ & \xrightarrow{K_{SS}^{NR}} & \\ R_{NR} & \longrightarrow & (30\text{–}51)_{NR} \end{array} \qquad (18)$$

where R_{NR} is the non-random conformation in reduced BPTI. The experimental data given above provide the values for

$$K_{SS}^{U} = 0.48 \text{ M} \qquad (19)$$

$$K_{conf}^{I} = 5.5 \qquad (20)$$

The value for the stability of the 30–51 disulphide bond in the non-random conformation is not known, but its value in fully folded BPTI is 1.7×10^3 M [11]. If this value applies here,

$$K_{conf}^{R} = \frac{K_{SS}^{U}}{K_{SS}^{NR}} K_{conf}^{I} = \frac{0.48 \text{ M}}{1.7 \times 10^3 \text{ M}} 5.5 \qquad (21)$$

$$K_{conf}^{R} = 1.6 \times 10^{-3} \qquad (22)$$

Therefore, the non-random conformation favouring the (30–51) intermediate would be expected to be populated in only 0.16% of reduced BPTI under normal conditions.

2.2. *Ribonuclease A*

The overall intramolecular rate for forming the first disulphide bonds in reduced ribonuclease A is similar to that observed in BPTI and about what would be expected for a random coil [53,54]. All Cys residues participate in forming the first disulphides. The resulting spectrum of one-disulphide intermediates is approximately random [54], and at least the majority of the intermediates appear to be fully unfolded [54–57]. Therefore, there is even less evidence for non-random conformation linked to disulphide formation in reduced ribonuclease A than in reduced BPTI.

3. Discussion

3.1. *Non-random conformation in reduced proteins*

Non-random conformation is clearly responsible for the predominant, and kinetically important, (30–51) one-disulphide intermediate in refolding of reduced BPTI. This non-random conformation is also likely to be present in the fully reduced protein, lacking the 30–51 disulphide bond, but only 0.16–5% of the time. Conse-

quently, it would not contribute significantly to the bulk properties of the reduced protein. Any non-random conformation favouring specific disulphide bonds in reduced ribonuclease A is even less significant.

Whatever other conformations exist in reduced BPTI and ribonuclease A, they must not favour proximity of any pairs of Cys residues, whether native-like or not. Consequently, these observations are untenable with suggestions that reduced BPTI [22] and reduced ribonuclease A [58,59] are conformationally or energetically close to their native-like conformations. On the basis of its slight enzymatic activity [58] and its interaction with anti-native antibodies [59], reduced ribonuclease A has been suggested to be in the native-like conformation, respectively, 0.04–1.5% and 6% of the time. One disulphide formed in reduced ribonuclease A is estimated by the classical theory to decrease its conformational entropy on average by a factor of 109 [60], so any ribonuclease A molecules having just one of the native disulphides should have a native-like conformation from 4% to over 99% of the time, yet there are no indications of such molecules [54–57]. Similarly, the dimensions of reduced BPTI measured by fluorescence energy transfer have been taken to indicate that nearly all the molecules are close to being native-like. If this were so, intermediates (30–51), (14–38) and (5–55) should predominate and have stable native-like conformations.

3.2. Relevance of non-random conformations for protein folding

Folding of reduced BPTI and ribonuclease require disulphide formation, yet it seems clear that any non-random conformations present in the bulk of these molecules do not favour disulphide formation and are therefore unlikely to have a role in productive folding. Unfolded proteins will acquire non-random conformations when placed in solvent conditions that favour any particular type of interaction, especially hydrogen bonding [61–63]. Yet, whenever the relevance for folding of such conformations has been examined, the non-random unfolded proteins have been found to fold at the same rate as apparently fully unfolded protein, under identical folding conditions [62–65]. The reason for this is that unfolded proteins are very mobile conformationally (ignoring the special case of slowly interconverted peptide bond isomers [66]), and all possible conformations interconvert rapidly on the time scale of complete folding; such rapid equilibration has been observed directly [23,24]. Any tendency for certain conformations to be favoured energetically will aid rapid equilibration of all possible conformations, and lead to all the molecules folding by the same pathway, or a limited sub-set of pathways. This is required by the simple kinetics of folding that are usually observed experimentally [67].

In summary, there is no evidence that any non-random conformations present in the bulk of unfolded proteins have any kinetic significance for folding. Even the α-helix at the amino-terminus of ribonuclease A that is exceptionally stable in the unfolded protein [29] is not finally incorporated into the folded conformation and stabilised until the very last detectable step [68].

That is not to say that all non-random conformation in the unfolded protein is not significant. Even if it is barely detectable, such as that favouring the 30–51 disulphide bond in reduced BPTI, it could be significant for folding if present at levels greater than would be expected in a truly random coil. It will be significant for folding if it is linked to other interactions and conformations that ultimately produce complete folding. Just as disulphides participate in folding by stabilising the non-random conformations that stabilise them (fig. 1) and the other disulphides of the final conformation, so other individually weak interactions will stabilise certain conformations and further interactions that eventually will produce a cooperative, fully folded structure [10].

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