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Enthalpic and entropic consequences of the removal of disulfide bridges in ribonuclease A

Giuseppe Graziano[a,b,*], Francesca Catanzano[c], Eugenio Notomista[d]

[a]*Department of Chemistry, University of Naples, Federico II, via Mezzocannone, 4-80134 Naples, Italy*
[b]*Faculty of Science, University of Sannio, via Port'Arsa, 11-82100 Benevento, Italy*
[c]*Faculty of Environmental Sciences, Second University of Naples, via Vivaldi, 81100 Caserta, Italy*
[d]*Department of Biological Chemistry, University of Naples, Federico II, via Mezzocannone, 16-80134 Naples, Italy*

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Abstract

In this study a careful analysis of the enthalpic and entropic effects associated with the removal of each of the four disulfide bridges in ribonuclease A is accomplished. The fundamental role of disulfides for the stability of the native structure is emphasized by the strong decrease in denaturation temperature, at least 20°C. In contrast to the traditional belief, the destabilization in two out of four mutant forms is driven by enthalpic factors, related to the loss or weakening of several van der Waals interactions among side-chains in the protein close-packed interior. Theoretical relations, derived in the assumption that the insertion of a disulfide does affect only the entropy of the denatured state by lowering its conformational freedom, prove to be unable to reproduce and explain the experimental results. Such failure is rationalized by taking into account the complexity of the protein structure in both the native and denatured states, and the large variety of stabilizing and destabilizing interactions involved.

Keywords: Disulfide bridge; Thermal unfolding; Conformational entropy; Thermodynamic stability

1. Introduction

The role played by disulfide bridges in the stabilization of the native structure of globular proteins is an important topic, not entirely addressed hitherto [1,2]. In the last few years, thanks to site-directed mutagenesis, it has been possible to directly compare the stability and structure of the wild-type protein with that of mutant forms lacking one or more disulfide bridges [3–6]. In addition to the removal, the insertion of novel disulfides has also been attempted in several proteins [7–10]. On the basis of these studies, Betz published a general and thorough review [11], emphasizing that most of them suffer from incomplete thermodynamic analysis. In fact, for a complete thermodynamic characterization of the protein stability, it is necessary to calculate the stability curve [12], the trend of the denaturation Gibbs energy change as a function of temperature, $\Delta_d G$ versus T.

Recently, an interesting paper has been published on this subject. Raines and co-workers [13] reported on the thermal stability and enzymatic activity of four variants of ribonuclease A (RNase A), each lacking one of the four disulfide bridges of the parent enzyme. In other words, each mutant does possess only three disulfide bridges and proves to be enzymatically

* Corresponding author. Tel.: +39-081-2536608; fax: +39-081-5527771.
E-mail address: graziano@unisannio.it (G. Graziano).

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active. The last property unequivocally demonstrates that all the four mutants have a tertiary structure closely similar to that of wild-type protein. The DSC measurements performed emphasize that the removal of a disulfide bridge destabilizes dramatically the folded structure because the denaturation temperature decreases of, at least, 20°C. However, Raines and co-workers did not perform a complete thermodynamic analysis of the consequences of disulfide removal. This task is accomplished in the present study.

2. Theoretical approaches

A large number of experimental investigations and theoretical models have demonstrated that the temperature-induced denaturation of small globular proteins is a reversible two-state N ⇔ D transition [14–16]. In other words, it has to be considered an intramolecular first-order phase transition, in which each protein molecule behaves as a single cooperative unit, with only two accessible thermodynamic states. In the following we use the term N-state to indicate the native folded structure of a globular protein, and the term D-state to indicate the ensemble of denatured unfolded conformations. According to the original hypothesis by Flory [17] and Schellman [18], the introduction of a disulfide bridge into a random coil polypeptide chain, considered a good model of D-state, should raise its Gibbs energy by lowering the conformational entropy. As a consequence, a disulfide bridge should stabilize the N-state of a globular protein by destabilizing entropically the D-state: the cross-link restricts the random coil chain to sampling fewer conformational states than if the cross-link did not exist. The N-state has always only one accessible conformation regardless of having or not a cross-link; on the contrary, the number of accessible conformations for the D-state strongly depends on the presence of a cross-link. Therefore, the introduction of a cross-link affects only the entropy of the D-state, leaving the entropy of the N-state unchanged. Also the energetic content of the two states is considered not to be affected.

Several authors [17–20] derived equations based on the polymer theory to estimate the magnitude of the entropy decrease, by calculating the ratio of the number of conformational states available to the chain containing the cross-link, to the number available if the chain does not contain the cross-link (i.e. the ensemble of all possible conformations). Such a ratio corresponds to the probability that the ends of a polymer chain simultaneously reside in the same volume element ΔV. If the chain is described by the random flight model in the gaussian approximation [21], the entropy decrease proves to be

$$\Delta S_{\mathrm{conf}} = R \ln \left[\Delta V \left(\frac{3}{2\pi b^2 N} \right)^{3/2} \right] \tag{1}$$

where R is the gas constant, N the number of statistical segments in the loop, and b the average length of a statistical segment, fixed at 3.8 Å for the C^{α}–C^{α} distance in polypeptide chains, as a result of the planar trans form of the peptide group. The open question is the value to assign to ΔV. Schellman [18] considered that the ends must reside in a region of volume $\Delta V = 2.83$ Å^3, whereas Poland and Scheraga [19] assumed a region of volume $\Delta V = 5.17$ Å^3. The above estimates are too small for the case where two –SH groups react to form a disulfide bridge. Indeed, according to the study of Thornton [22], the distance of closest approach of two –SH groups is about 4.8 Å and the volume of a sphere with a diameter of 4.8 Å is 57.9 Å^3. By using this value for ΔV into Eq. (1), Pace and co-workers [23] obtained the following relation:

$$\Delta S_{\mathrm{conf}} = -8.8 - 1.5R \ln N_{\mathrm{res}} \quad \text{in J K}^{-1}\,\text{mol}^{-1} \tag{2}$$

where N_{res} is the number of residues in the loop formed by the disulfide bridge. Harrison and Sternberg [24], in a careful analysis of the occurrence and connectivity of disulfides in a large set of globular proteins, recently, proposed $\Delta V = 29.65$ Å^3 and obtained:

$$\Delta S_{\mathrm{conf}} = -14.4 - 1.5R \ln N_{\mathrm{res}} \quad \text{in J K}^{-1}\,\text{mol}^{-1} \tag{3}$$

In the following we will use the latter relationship. According to the original hypothesis by Flory and Schellman, the decrease in conformational entropy of the D-state leads to the following variation in the denaturation Gibbs energy change:

$$\Delta\Delta_{\mathrm{d}}G = -T\Delta S_{\mathrm{conf}} \tag{4}$$

This is a positive quantity, thus implying that the

introduction of a disulfide into the polypeptide chain stabilizes the N-state. Thornton pointed out that 49% of the disulfides occurring in globular proteins are separated by less than 24 residues and that 15 residues is the average separation [22]. This datum indicates that the loop length has not been maximized in globular proteins by evolution. In this regard, however, it should be noted that Harrison and Sternberg devised a theoretical treatment, based on multivariate gaussian distributions, to assess the optimal arrangements of multiple cross-links in terms of conformational entropy loss of the polymer chain [24]. Counter-intuitively, but in agreement with globular protein data, they found that small loop lengths are often optimal for the insertion of an additional cross-link in a chain already containing cross-links.

In any case, using Thornton's result, a disulfide bridge, according to Eqs. (3) and (4), should contribute, on the average, 14 kJ mol^{-1} to the conformational stability of a globular protein at room temperature. However, disulfides appear to be poor choices to achieve an extreme stability against thermal denaturation because cystine may oxidize at high temperature [25]. In fact, the study of proteins from thermophilic microorganisms suggests that electrostatic interactions, and ion pairs in particular, should be the main responsible of the extra-stability [26–28].

It has to be noted that, in the random flight model, the polymer is considered as an assembly of identical monomers that do not occupy a volume. However, in a real polymer, each monomer does possess a volume and certain conformations of the chain are excluded due to steric overlap. This is the well-known excluded volume effect [21]. Chan and Dill [29,30], by performing computer simulations of self-avoiding chains on 2D square and 3D cubic lattices, investigated how the excluded volume affects the probability of loop formation. They found that, by taking into account the excluded volume effect, the probability of loop formation decreases, and the characteristic exponent for a single loop increases from 1.5 (i.e. $N^{3/2}$ in Eq. (1)) to a value in the range 1.8–2.4.

In spite of the uncertainty associated with the choice of ΔV and the need to properly account for the excluded volume effect, such an approach leads to the conclusion that: (a) disulfide bridges stabilize the N-state by lowering the conformational entropy of the D-state; (b) the stabilization increases on lengthening the size of the loop. Clearly, in the case of the removal of a disulfide bridge, the above equations remain valid, but the signs in Eqs. (2) and (3) have to be reversed.

3. Analysis of experimental data and discussion

The structure of RNase A is characterized by three α-helical segments, spanning residues 3–13, 24–34 and 50–60, respectively; and two anti-parallel β-sheets, that give rise to the V-shaped motif characteristic of the tertiary fold of pancreatic ribonucleases [31]. The first β-sheet has three strands, spanning residues 42–48, 79–87 and 97–104, respectively; the second β-sheet is less regular, with two long strands, comprising residues 105–113 and 114–124, and two short strands, comprising residues 61–64 and 71–75. In addition, the structure of RNase A is characterized by the presence of four disulfides, whose fundamental role was emphasized by the classic experiments of Anfinsen and colleagues [32]. RNase A unfolds completely upon breakage of the four disulfides, but refolds as the disulfides are re-formed. These findings were a cornerstone for the validity of the so-called ''thermodynamic hypothesis'' in protein folding [33,34].

The disulfide Cys65–Cys72 closes an external and exposed loop, while the disulfide Cys40–Cys95 connects the loop following the second α-helix to the loop between two strands of the first β-sheet. The disulfide Cys26–Cys84 connects the second α-helix to the second strand of the first β-sheet, while the disulfide Cys58–Cys110 connects the third α-helix to the first long strand of the second β-sheet. Therefore, since each of the latter disulfide bridges connects two rather rigid secondary structure elements, it can be surmised that they should play a very important role for the stability of the tertiary fold of RNase A.

In general, disulfide bridges in globular proteins are buried in the core to avoid contact with water [22,35]. This rule is confirmed in the case of RNase A: the accessible surface areas of the cystine side-chains, calculated from the crystal structure of the protein (Protein Data Bank entry code 7rsa [31]), prove to be negligibly small. This is a manifestation of the hydrophobic character of disulfides [36]. By recognizing this important feature, Raines and co-workers [13] decided to replace cysteine residues with alanine

residues, which have a small nonpolar side-chain. In other words, a pair of alanine residues seems to be the most conservative replacement for a disulfide bridge. In a previous study, Scheraga and co-workers replaced the disulfides in RNase A with pairs of serine residues [37]. Such a substitution, although conservative of the side-chain size (i.e. the side-chain volumes are 39.7 $Å^3$ for Cys, 30.4 $Å^3$ for Ser, and 26.3 $Å^3$ for Ala, as determined by Chothia and co-workers on a large set of well-resolved protein structures [38]), can lead to an overestimation of the stability provided by disulfides. In fact, the serine side-chain is polar and its burial in the hydrophobic interior should be destabilizing.

The thermal stability of RNase A and the four mutant forms was investigated performing DSC measurements at pH 6.0, 30 mM acetate buffer and 100 mM NaCl [13]; the corresponding results are collected in Table 1. For all the proteins, the process proved to be reversible, according to the reheating criterion, and well represented by the two-state N ⇔ D transition model, according to the closeness of the calorimetric and van't Hoff enthalpy changes [39]. It results that the denaturation temperature T_d decreases from 62.1°C for RNase A, to 42.7°C for C65A–C72A-RNase A, 39.3°C for C40A–C95A-RNase A, 26.8°C for C26A–C84A-RNase A, and 26.1°C for C58A–C110A-RNase A. These values strongly emphasize that the thermal stability of the folded structure of RNase A depends substantially on the disulfide bridges. The removal of the disulfide bridge encompassing the smallest loop Cys65–Cys72 causes the smallest destabilization, but, in any case, it amounts to about 20°C. Removal of the two disulfides connecting two secondary structure elements of the RNase A tertiary fold, causes a decrease in T_d of about 36°C.

The validity of the two-state N ⇔ D model implies that, for C26A–C84A-RNase A and C58A–C110A-RNase A, half of the molecules are folded at 25°C and half are unfolded. Moreover, DSC results, in the usual assumption that the effects of mutations are additive, mean that it is not possible to prepare a mutant form of RNase A lacking two disulfide bridges: all the molecules would be unfolded at room temperature. Clearly, this hypothesis should be tested experimentally, and work is in progress in such direction.

On the other hand, it is worth noting that the introduction of an extrinsic cross-link in RNase A leads to a significant increase in thermal stability. In fact, Scheraga and co-workers prepared a cross-linked derivative of pancreatic ribonuclease, K7-dinitrophenilene-K41-RNase A, that, at pH 2.0, showed a denaturation temperature 25°C higher than that of the unmodified RNase A under the same conditions [20].

In order to perform a correct and meaningful comparison among the thermodynamic stability of the five ribonucleases, and to estimate the enthalpic and entropic effects associated with the removal of each disulfide, it is necessary to calculate the functions $\Delta_d H$, $\Delta_d S$ and $\Delta_d G$ at the same temperature [40,41], by means of the following equations:

$$\Delta_d H(T) = \Delta_d H(T_d) + \Delta_d C_p (T - T_d) \quad (5)$$

$$\Delta_d S(T) = \left[\frac{\Delta_d H(T_d)}{T_d}\right] + \Delta_d C_p \ln\left(\frac{T}{T_d}\right) \quad (6)$$

$$\Delta_d G(T) = \Delta_d H(T) - T\Delta_d S(T) \quad (7)$$

These are exact assuming $\Delta_d C_p$ temperature independent [39]. Their application in this case is strictly correct because the denaturation process is a reversible two-state transition for all the proteins. In order to

Table 1
Thermodynamic parameters of the temperature-induced denaturation of the five ribonucleases, obtained from DSC measurements at pH 6.0, 30 mM acetate buffer and 100 mM NaCl[a]

	T_d (°C)	$\Delta_d H(T_d)$ (kJ mol^{-1})	$\Delta_d S(T_d)$ (kJ K^{-1} mol^{-1})	$\Delta_d H_v H(T_d)$ (kJ mol^{-1})
RNase A	62.1	476	1.42	476
C65A–C72A-RNase A	42.7	384	1.22	376
C40A–C95A-RNase A	39.3	323	1.03	313
C26A–C84A-RNase A	26.8	294	0.98	282
C58A–C110A-RNase A	26.1	190	0.64	199

[a] See reference [13].

Table 2
Comparison between the denaturation enthalpy, entropy and Gibbs energy changes of the five ribonucleases, evaluated at the same reference temperature, $T = 25^{\circ}C$, by means of Eqs. (5)–(8)[a]

	$\Delta_d H$	$T\Delta_d S$	$\Delta_d G$	$\Delta\Delta_d H$	$T\Delta\Delta_d S$	$\Delta\Delta_d G$
RNase A	297.2	254.6	42.6	–	–	–
C65A–C72A-RNase A	298.2	279.0	19.2	1.0	24.4	−23.4
C40A–C95A-RNase A	253.6	240.3	13.3	−43.6	−14.3	−29.3
C26A–C84A-RNase A	284.6	282.7	1.9	−12.6	28.1	−40.7
C58A–C110A-RNase A	184.0	183.2	0.8	−113.2	−71.4	−41.8

[a] All the values are in kJ mol^{-1} units.

perform calculations, we fix $\Delta_d C_p = 4.8$ kJ K^{-1} mol^{-1} for the five ribonucleases, as determined by Pace and co-workers for RNase A [42]. This assumption can be justified. Since all the proteins are enzymatically active [13], they should possess very similar tertiary structures (i.e. the side-chains of His12, Lys41 and His119 have to be correctly positioned for the exploitation of the catalytic activity), and, as a consequence, should have similar $\Delta_d C_p$ values [43]. In addition, by selecting $T = 25^{\circ}C$, the extrapolation range is not large for the mutant forms. The values of $\Delta_d H$, $\Delta_d S$ and $\Delta_d G$ calculated at 25°C are reported in the second, third and fourth columns, respectively, of Table 2. Moreover, we calculate the functions:

$$\Delta\Delta_d X = \Delta_d X(\mathrm{mt}, 25^{\circ}C) - \Delta_d X(\mathrm{wt}, 25^{\circ}C) \quad (8)$$

where X stands for H, S and G, while mt and wt mean mutant and wild-type, respectively; the corresponding values are listed in the fifth, sixth and seventh columns, respectively, of Table 2. It is worth noting that negative values of $\Delta\Delta_d G$ mean that the mutant form is less stable than the parent protein, negative values of $\Delta\Delta_d H$ indicate that the enthalpic factors have a destabilizing effect on the folded structure, whereas negative values of $T\Delta\Delta_d S$ indicate that the entropic factors have a stabilizing effect on the folded structure.

Clearly, the values of $\Delta\Delta_d G$ are large and negative, but we are principally interested in the enthalpic and entropic contributions. The estimates reported in Table 2 point out that the destabilization is purely entropic only in the case of C65A–C72A-RNase A, and it is dominated by entropic effects also in the case of C26A–C84A-RNase A. On the contrary, the destabilization is dominated by enthalpic effects in the case of C40A–C95A and C58A–C110A mutants; in such cases the entropic effects tend to stabilize the folded structure (i.e. the $T\Delta\Delta_d S$ estimates are negative), in complete contrast with the expectation based on Eqs. (3) and (4). At first sight, negative values of $T\Delta\Delta_d S$ may seem strange, being at variance with theoretical ideas, however, a reliable explanation can be provided. The loss of a covalent cross-link can increase the local flexibility of the polypeptide chain in the N-state to such an extent that the entropy gain upon denaturation is smaller than that of the protein possessing the covalent cross-link.

This reasoning suggests that the analysis performed by Tidor and Karplus [44] was on the right track: the insertion or removal of a disulfide bridge does affect the conformational entropy of both the N-state and D-state. This implies that the effects are largely dependent on the specific structural features of the protein region involved. Vice versa, the idea that the enhancement in thermodynamic stability induced by disulfide insertion should be caused by an enthalpic destabilization of the D-state owing to its compactness, advanced by Doig and Williams [45], seems to be entirely wrong. In all probably, these authors used a too small set of globular proteins for their analysis, and overestimated the ability of a disulfide to decrease the accessible surface area of the D-state.

On the other hand, the finding of an enthalpic destabilization in three out of four mutant forms deserves a further comment. Since the tertiary structure of globular proteins is characterized by a close-packed core, resembling a crystalline solid [38,46–48], the removal of disulfide bridges should cause the loss or weakening of several van der Waals interactions among buried side-chains. This gives rise to a significant decrease in the energetic content of the N-state, only partially counterbalanced by a corresponding increase in conformational freedom.

Experimental data prove that the disulfide Cys40–Cys95 contributes to the stability of RNase A less than the disulfides Cys26–Cys84 and Cys58–Cys110, even though the three loops are similar in size. This finding again indicates the failure of Eqs. (3) and (4), but can be explained by considering the specific nesting of disulfides bridges in the primary structure of RNase A and their location in the tertiary structure of the protein. In fact, according to the analysis of Karpeisky and colleagues [49], the disulfide bridges Cys26–Cys84 and Cys58–Cys110 are each comprised in the two main hydrophobic nuclei of RNase A, whereas the disulfide Cys40–Cys95 is located in a small hydrophobic cluster. A hydrophobic nucleus is defined as the most compact part of a nonpolar region of the protein structure, constituted by amino acid residues having not less than two nonpolar neighbors at a cutoff distance of 4.5 Å, provided that at least three of them contact each other to form an interacting triangle [49]. Therefore, with respect to the side-chain of an average residue, those belonging to a hydrophobic nucleus have higher number of contacts, smaller temperature factors and lower water accessibility. Accordingly, hydrophobic nuclei should be considered as relatively rigid parts of a protein, having structural and functional importance. In fact, they prove to be the most conserved regions in pancreatic ribonucleases [50].

However, the estimates reported in Table 2 indicate that enthalpic factors are destabilizing for both C26A–C84A-RNase A and C58A–C110A-RNase A, whereas entropic factors are destabilizing in the first case, but stabilizing in the second one. This is a puzzling observation because both disulfides connect an α-helix to a β-strand, and are deeply buried in hydrophobic nuclei. Actually, a careful inspection of RNase A structure reveals that, in the region around the bridge Cys26–Cys84, there are several H-bonds whose strength and directionality could be sufficient to avoid large local fluctuations, even in the absence of the disulfide. Such H-bonds are not present in the region around the bridge Cys58–Cys110. This is a strong confirmation of the very subtle balance between stabilizing and destabilizing interactions operative in globular proteins [2,14].

The conformational entropy changes due to the removal of disulfides in RNase A, calculated by means of Eq. (3), upon sign reversal, and the corresponding contributions to the protein stability at 25°C, calculated by means of Eq. (4), are reported in the third and fourth columns, respectively, of Table 3. A direct comparison between the experimental $\Delta\Delta_d G$ values and the $-T\Delta S_{conf}$ estimates indicates that Eqs. (3) and (4) are unable to predict the effect of disulfide removal in RNase A. In order to confirm this point, we use the following relationship:

$$T_{d'} = \frac{\Delta_d H(T_d, \mathrm{wt})}{[\Delta_d S(T_d, \mathrm{wt}) + \Delta S_{conf}]} \quad (9)$$

to estimate the denaturation temperatures of the various mutant forms of RNase A. The calculated $T_{d'}$ values, reported in the fifth column of Table 3, prove to be strongly different from the experimental denaturation temperatures listed in the second column of Table 1. The failure of Eq. (3) is not surprising because: (a) short polypeptide chains deviate substantially from the gaussian behavior assumed in the derivation of that equation; (b) the cross-links investigated overlap other cross-links in the molecule, a situation ignored in the theoretical treatment; (c) the role played by energetic factors in the close-packed interior of globular proteins is not considered at all.

Table 3
Conformational entropy change due to the removal of disulfide bridges in RNase A, calculated by means of Eq. (3), upon sign reversal, and the corresponding contribution to the protein stability at 25°C, calculated by means of Eq. (4)[a]

	N_{res}	ΔS_{conf} (J K^{-1} mol^{-1})	$-T\Delta S_{conf}$ (kJ mol^{-1})	$T_{d'}$ (°C)
C65A–C72A-RNase A	8	40.3	−12.0	52.8
C40A–C95A-RNase A	56	64.6	−19.3	47.5
C26A–C84A-RNase A	59	65.3	−19.5	47.3
C58A–C110A-RNase A	53	63.9	−19.1	47.6

[a] The values of $T_{d'}$ are calculated by means of Eq. (9).

4. Conclusion

The original hypothesis by Flory and Schellman [17,18], although appealing for its simplicity, proves to fail because it does not take into account the complexity of the protein structure and the intricate interplay between stabilizing and destabilizing interactions. For instance, the assumption that the D-state of a globular protein can be considered a random coil is certainly an oversimplification, as well emphasized by Dill and Shortle [51]. On the other hand, one cannot neglect that the extreme cooperativity of the N-state is due to the tight and unique packing of side-chains in the protein interior as in a jigsaw puzzle [52]. Therefore, even though the excluded volume effect and the connectivity of disulfides in the polypeptide chain were correctly taken into account to calculate the conformational entropy loss of the D-state, a theoretical approach, which assumes the N-state be unaffected by the removal or insertion of a cross-link, would be on the wrong track. The latter assumption is absolutely not justified by the experimental data of Raines and co-workers on the mutant forms of RNase A [13], and has to be considered, in general, not correct. In conclusion, the above analysis strengthens the rule that experiments are the ultimate arbiter of theoretical models.

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