Biophysical Chemistry 141 (2009) 21–28

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

The effect of additional disulfide bonds on the stability and folding of ribonuclease A

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ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 11 December 2008
Accepted 13 December 2008
Available online 30 December 2008

Keywords:
Ribonuclease A
Thermodynamic stability
Protein folding
Disulfide bond
Protein engineering

ABSTRACT

The significant contribution of disulfide bonds to the conformational stability of proteins is generally considered to result from an entropic destabilization of the unfolded state causing a faster escape of the molecules to the native state. However, the introduction of extra disulfide bonds into proteins as a general approach to protein stabilization yields rather inconsistent results. By modeling studies, we selected positions to introduce additional disulfide bonds into ribonuclease A at regions that had proven to be crucial for the initiation of the folding or unfolding process, respectively. However, only two out of the six variants proved to be more stable than unmodified ribonuclease A. The comparison of the thermodynamic and kinetic data disclosed a more pronounced effect on the unfolding reaction for all variants regardless of the position of the extra disulfide bond. Native-state proteolysis indicated a perturbation of the native state of the destabilized variants that obviously counterbalances the stability gain by the extra disulfide bond.

1. Introduction

The folding process of proteins starts with a hydrophobic collapse or other local events at so-called 'nuclei' [1], 'seeds' [2], or 'initiation sites' [3] and proceeds stepwise to the natively folded protein [4,5]. In contrast to the successive protein folding process, protein unfolding is a highly cooperative reaction. Consequently, intermediates are much less frequently populated here [6]. Due to the principle of microscopic reversibility, however, unfolding is a stepwise process as well, which is postulated to start at confined regions of the tertiary structure of the native protein molecule [7]. In fact, numerous studies accumulate evidence for the existence of suchlike regions, which accordingly were termed 'unfolding region' [8,9], 'critical region' [10], 'unfolding nucleation site' [11], or similar.

The rate constants of the folding (k_f) and unfolding (k_U) reaction, both of which determine the respective free energy of activation $\Delta G_f^{\ddagger}$ and $\Delta G_U^{\ddagger}$ and, thus, the Gibbs free energy ΔG, provide information on the contribution of these reactions to the thermodynamic stability [12]. The comparison of proteins with their chemically modified or genetically engineered variants yields information on the involvement of specific residues in the folding or unfolding reaction. This approach has resulted in the Φ-value ($\Delta\Delta G^{\ddagger}/\Delta\Delta G$) analysis by Fersht and coworkers to evaluate the native-like arrangement of specific residues in the transition state [13,14]. As a consequence, modifications in the folding region should influence ΔG by changing k_f ($\Delta G_f^{\ddagger}$) whereas modifications in the unfolding region should influence ΔG by changing k_U ($\Delta G_U^{\ddagger}$).

Proteins from thermophilic organisms structurally differ from their mesophilic homologues by an increase in the number of native contacts (increase in ΔG by stabilizing the native state N) or by the introduction of proline residues and disulfide bonds. According to the chain entropy model [15–17], introduced proline residues and disulfide bonds are considered to increase ΔG by an entropic destabilization of the unfolded state [18–20]. However, particularly introduced disulfide bonds may affect the native state as well since they might prevent unfolding by tethering the native protein structure [21].

Bovine pancreatic ribonuclease A (RNase A) is one of the most thoroughly studied model proteins concerning the protein folding problem. The folding region of RNase A had been postulated for residues 106–118 (Fig. 1; [22]) and its importance for the folding and stability of the RNase A molecule has been confirmed by mutagenesis studies [23] or replacement of *cis*Pro114 by the *cis*-locked β-turn mimic 5′,5′-dimethylproline [24]. As for the unfolding process, the section from the C-terminal end of helix II (Lys31) through the first β-sheet strand (Phe46) became susceptible first to proteolytic attack by thermolysin and trypsin under denaturing conditions [25] and several residues in this region showed a faster H–D exchange than that of global unfolding [26,27]. The designation of this region as unfolding region of RNase A was confirmed by both computer simulations and studies using either the glycosylated variant of RNase A, RNase B, or genetically engineered RNase A variants [28–30].

Abbreviations: AUAA, 6-carboxyfluorescein-dArU(dA)$_2$-6-carboxytetramethylrhodamine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GdnHCl, guanidine hydrochloride; ONC, onconase; RNase, ribonuclease.

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doi:10.1016/j.bpc.2008.12.005

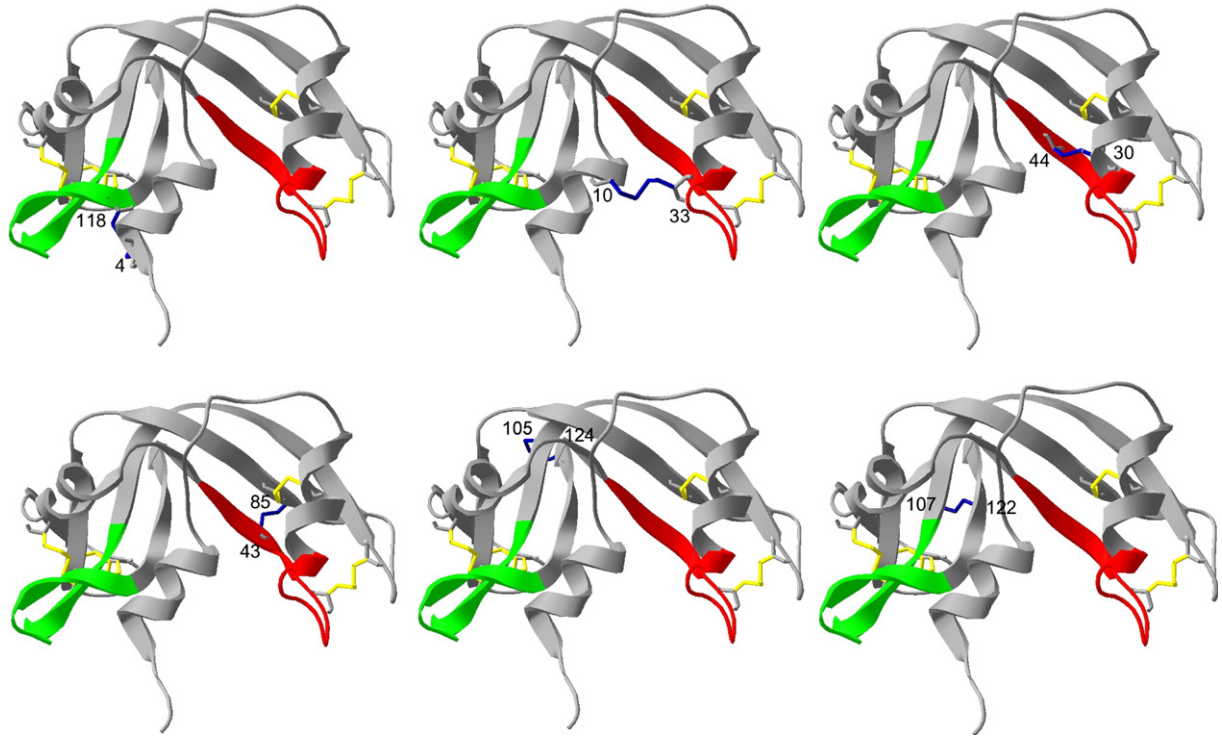

Fig. 1. Tertiary structure of RNase A with the introduced disulfide bonds. The model (7rsa) was taken from the Brookhaven protein data bank and drawn with Swiss pdb-Viewer v3.7. In addition to the native disulfide bonds (yellow), the position of the respective additional disulfide bond 4–118, 10–33, 30–44, 43–85, 105–124, or 107–122 is indicated in blue. Furthermore, the proposed folding region (residues 106–118, [22]) and unfolding region (residues 31–46, [25]) are indicated in green and red, respectively, labeling R10C/R33C-, M30C/N44C-, and V43C/R85C-RNase A as unfolding variants and A4C/V118C-, H105C/V124V-, and I107C/A122C-RNase A as folding variants (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

RNase A contains four disulfide bonds that tether the protein molecule (Fig. 1) and considerably contribute to its thermodynamic stability. Replacement of single cystines diminished the transition temperature T_m by about 20–36 °C [31,32]. In reversal, an 1,5-difluoro-2,4-dinitrobenzene cross-link between Lys7 and Lys41 [33] increased T_m by 25 °C [34] or 12.5 kJ mol^{-1} [35], which was almost exclusively caused by a deceleration of the unfolding reaction ($\Delta\Delta G_U^{\ddagger} \approx 11$ kJ mol^{-1}, [35]). Introduction of an additional disulfide bond by genetic engineering into human pancreatic RNase 1 (R4C/V118C-RNase 1) increased ΔG by 9 kJ mol^{-1} [36] and the analog disulfide bond in A4C/G88R/V118C-RNase A increased T_m by 4.8 °C in comparison to G88R-RNase A [37]. The influence of the extra disulfide bond on the folding behavior of the RNase, however, has not been studied. Interestingly, in onconase (ONC), a homolog of RNase A with strongly reduced k_U in comparison to RNase A [38], three out of the four disulfide bonds of RNase A are conserved. The forth disulfide bond, which tethers the C-terminus of ONC to the protein body, significantly contributes to the stability of ONC as its replacement by a pair of alanines reduces the stability of ONC by 32 kJ mol^{-1} due to an increase in k_U and a decrease of k_f [38].

In a recent study [39], we have investigated the thermal unfolding of A4C/V118C- and V43C/R85C-RNase A as variants with additional cross-links in the folding and unfolding region of RNase A, respectively. In contrast to the expectation from the chain entropy model, the increase in stability (ΔT_m=+4.9 °C and +2.2 °C, respectively) was found to result mainly from a decrease in k_U in both variants. More recently, Pradeep et al. [40] have studied the same variants in guanidine hydrochloride (GdnHCl) and confirmed the decrease of k_U observed under thermal denaturation. While no change in k_f was found for V43C/R85C-RNase A (confirming the expectations from thermal denaturation), folding of A4C/V118C-RNase A was found to be decelerated by almost the same extent as k_U. Even though these counteracting effects should compensate for any effect on ΔG, T_m was found to be increased by about 6 °C.

Here we extend our previous studies by four additional variants modifying both the folding and unfolding region of RNase A. Equilibrium unfolding studies are correlated to folding/unfolding kinetics to dissect the effect of the introduced disulfide bonds on the unfolded, transition, and native state, respectively. In contrast to the expectation, in all variants mainly the unfolding reaction is affected. The extent of the effect, however, strongly depends on the position of the newly introduced disulfide bond.

2. Materials and methods

2.1. Materials

Oligonucleotides and 6-carboxyfluorescein-dArU(dA)$_2$-6-carboxy-tetramethylrhodamine (AUAA) were from metabion international AG, Martinsried, Germany. RNase A was from Sigma, Taufkirchen, Germany, restriction enzyme *Dpn*I was from New England Biolabs, Frankfurt/Main, Germany, growth media were from Difco Laboratories, Detroit, MI, USA, and *Escherichia coli* (*E. coli*) strains XL-1 Blue and BL21(DE3) were from Stratagene, Heidelberg, Germany. All other chemicals were of purest grade commercially available.

2.2. Site-directed mutagenesis

The *rnase A* gene in pET-26b(+) [41] was modified by use of the QuikChange™ site-directed mutagenesis kit (Stratagene) to obtain the mutations A4C/V118C, R10C/R33C, M30C/N44C, V43C/R85C, H105C/V124C, and I107C/A122C. The mutations were introduced in two steps except for M30C/N44C (one step, see Table S-1) and verified by DNA sequencing according to Sanger et al. [42] using the SequiThermExcel™ LongRead™ DNA sequencing kit (Biozym, Hess. Oldendorf, Germany) and a Li-COR 4000 DNA-sequencer (MWG Biotech, Ebersberg, Germany).