Reduced-denatured ribonuclease A is not in a compact state

Angela Nöppert[a,*], Klaus Gast[a], Marlies Müller-Frohne[b], Dietrich Zirwer[b], Gregor Damaschun[b]

[a]*Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, D-13122 Berlin, Germany*
[b]*Humboldt-University at Berlin, Institute of Biology, Berlin, Germany*

Received 11 December 1995

Abstract Dynamic light scattering and circular dichroism experiments were performed to determine the compactness and residual secondary structure of reduced and by 6 M guanidine hydrochloride denatured ribonuclease A. We find that reduction of the four disulphide bonds by dithiothreitol at 20°C leads to total unfolding and that a temperature increase has no further effect on the dimension. The Stokes' radius of ribonuclease A at 20°C is R_s = (1.90 ± 0.04) nm (native) and R_s = (3.14 ± 0.06) nm (reduced-denatured). Furthermore, circular dichroism spectra do not indicate any residual secondary structure. We suggest that reduced-denatured Ribonuclease A has a random coil-like conformation and is not in a compact denatured state.

Key words: Ribonuclease A; Protein folding; Denaturation; Guanidine hydrochloride; Dynamic light scattering; Circular dichroism

1. Introduction

In order to understand the principles of protein folding, experiments regarding the relationship between secondary structure formation and compactness of the polypeptide chain are essential. It has been shown that different denaturing conditions (pH, temperature, chemical agents) may lead to different structural end points of denaturation. Many studies have reported that proteins denatured in high concentrations of aqueous guanidine hydrochloride (GuHCl) behave like random coils [1,2,3,4]. However, it is still unclear whether proteins in the presence of strongly denaturing agents show residual secondary structure and are more compact than predicted for a statistical polypeptide chain at the θ-point [1,2,3,4,5]. Contrary results were presented for ribonuclease A (RNase A). Tanford et al. [2] showed that the dependence of the intrinsic viscosity on the number of amino acids N_A in 6 M GuHCl is exactly as predicted for a randomly coiled polymer chain [6,7,8]. They determined the intrinsic viscosity of different proteins lacking disulfide bonds, including reduced RNase A and found:

$$[\eta] \propto N_A^{0.67}.$$

Based on the random coil model they calculated a root mean square end-to-end-distance $\langle r^2\rangle^{\frac{1}{2}}$ according to the relation [6,7,8]:

$$\langle r^2\rangle^{\frac{3}{2}} = N_A \cdot M_0 \cdot [\eta]/\Phi.$$

Here $\Phi = 2.1 \cdot 10^{23}$ and M_0 is the average molecular weight per monomer. For RNase A they obtained $\langle r^2\rangle^{\frac{1}{2}}$ = 10.1 nm. One can express the root mean square end-to-end distance as a power law in N_A:

$$\langle r^2\rangle^{\frac{1}{2}} = \gamma^{\frac{1}{2}} \cdot N_A^d$$

where d is a parameter that depends upon the chain conformation and γ is a constant. Miller and Goebel [1] calculated dimensions of unperturbed random coil polypeptides. They found:

$$\langle r^2\rangle = \gamma \cdot N_A$$

with 0.91 $(\text{nm})^2 \leq \gamma \leq 1.0$ $(\text{nm})^2$ which would mean 10.6 nm $\leq \langle r^2\rangle^{\frac{1}{2}} \leq$ 11.1 nm for reduced-denatured RNase A. Comparing these results shows that the value derived experimentally by Tanford et al. [2] is somewhat smaller than the one calculated by Miller and Goebel [1]. In the limit of infinite chain length the mean square end-to-end distance is related to the radius of gyration via [9]:

$$\langle R_G^2\rangle = \frac{\langle r^2\rangle}{6}.$$

For reduced-denatured RNase A this leads to R_G = 4.1 nm according to Tanford et al. [2] and 4.3 nm $\leq R_G \leq$ 4.5 nm according to Miller and Goebel [1]. Damaschun et al. [10] measured Stokes' radii R_s for different proteins, lacking disulfide bonds, denatured by 6 M GuHCl. Conversion of Stokes' radii R_s into radii of gyration R_G via $R_G = \rho \cdot R_s$ with ρ = 1.6 led to the scaling law:

$$\langle R_G^2\rangle = \frac{\gamma}{6} \cdot N_A$$

with γ = (1.17 ± 0.07) $(\text{nm})^2$. The radius of gyration R_G of reduced-denatured RNase A would therefore be: R_G = (4.9 ± 0.2) nm. Sosnick and Trewhella [5] performed small-angle X-ray scattering (SAXS) and Fourier transform infrared spectroscopy (FTIR) on RNase A. In contrast to the previous results summarized above they found a very small radius of gyration of: R_G = (2.41 ± 0.10) nm for reduced-denatured RNase A. They concluded that reduced-denatured RNase A is more compact than a random coil under θ-conditions and has a significant amount of regular secondary structure which was deduced from FTIR spectra. In addition they claim that reduced RNase A is not unfolded at 20°C but begins to unfold only at temperatures above 51°C. Haas et al. [11] used fluorescence lifetime measurements to study localized conformational changes. They observed an average distance of 6 nm

*Corresponding author. Fax: (49) (30) 94062548.

Abbreviations: GuHCl, guanidine hydrochloride; RNase A, ribonuclease A; SAXS, small-angle X-ray scattering; FTIR, Fourier transform infrared spectroscopy; DLS, dynamic light scattering; CD, circular dichroism; DTT, dithiothreitol.

SSDI 0014-5793(96)00048-8

between residues 1 and 50 for reduced-denatured RNase A and concluded that this is significantly smaller than expected for a random coil. They also suggested the presence of residual structure in the N terminal segment. Bierzynski and Baldwin [12] also found indication of local secondary structure in the N terminus of denatured RNase A. They performed proton nuclear magnetic resonance on the C-peptide (residue 1 to 13 of RNase A) and the S-peptide (residue 1 to 20 of RNase A) at 1°C in GuHCl. They observed that the α-helix found in the native protein remains stable in the C-peptide and S-peptide in the denatured protein.

In the following we contribute to resolve the discrepancy in the literature emphazising the special preparation of reduced RNase A. Dynamic light scattering (DLS) was performed in order to determine the Stokes' radius. Secondary structure was studied by measurements of the circular dichroism (CD).

2. Materials and methods

2.1. Materials

Bovine pancreatic RNase A was purchased from Sigma (type XII-A) and was used without further purification. Ultra pure GuHCl was obtained from ICN Biomedicals, Inc. Sample protein concentrations were determined spectrophotometrically using the absorption at 277 nm of: $A^{0.1\%}_{1\,cm} = 0.695$.

2.2. Preparation of reduced RNase A

2.2.1. Reduction of RNase A. RNase A was dissolved in 50 mM MES (pH 7.5) containing 6 M GuHCl in order to unfold the protein and hence make its disulfide bonds accessible for splitting. The reduction was performed at a protein concentration of about 1.5 to 2 mM by incubation with a forty fold excess of dithiothreitol (DTT) for 3 h at 22°C.

2.2.2. Isolation and stabilization of the reduced state of RNase A. Reduced RNase A was separated from the excess of DTT by gel filtration using a FPLC-Column (Pharmacia Desalting HR 10/10 Sephadex G25 Superfine). The column was equilibrated with 10 mM glycine/HCl buffer (pH 2.5) for a simultaneous decrease of the pH in order to stabilize the formed thiol groups of the protein.

2.2.3. Preparation of reduced RNase A for CD and DLS measurements. Shortly before the measurements, the according to sections 2.2.1. and 2.2.2. reduced RNase A was brought to the appropriate pH by buffer exchange using the FPLC Desalting Column equilibrated with the corresponding buffer. The buffer always contained 1 mM EDTA in order to prevent the thiol groups from reoxidation.

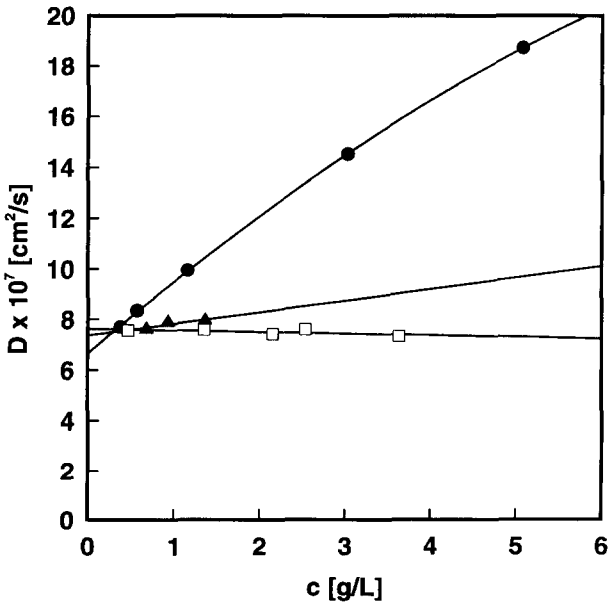

Fig. 1. Translational diffusion coefficient D versus concentration of RNase A at $T = 20°C$. (▲), 10 mM sodium acetate buffer, pH 4; (●), 10 mM glycine/HCl buffer, pH 2.5; (□), 50 mM MES buffer, pH 5.7. Curves (▲) and (□) were fitted linearly. A quadratic fit was done for (●). D_0 is found to be $D_0 = 6.64 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1} (●), $D_0 = 7.36 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1} (▲), $D_0 = 7.63 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1} (□).

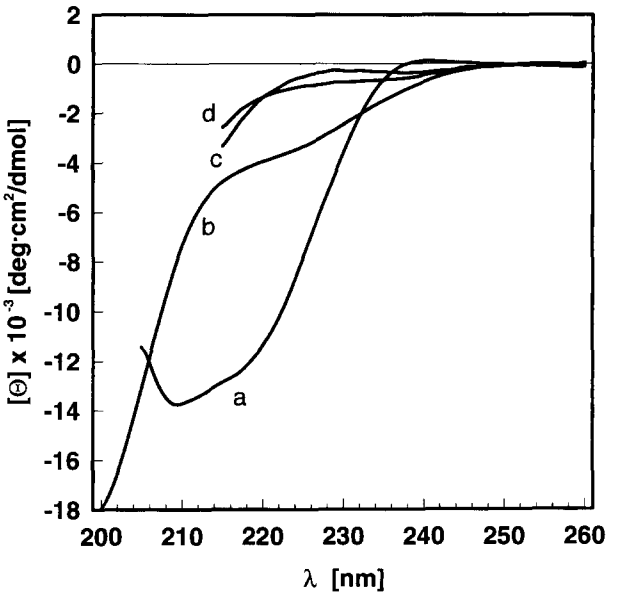

Fig. 2. CD spectra for (a) native RNase A, (b) reduced RNase A, (c) RNase A denatured by 6 M GuHCl, (d) reduced-denatured RNase A at $T = 20°C$ (compare Table 3).

2.3. DLS measurements

The laboratory built DLS apparatus was described elsewhere [13]. It mainly consists of an argon laser LEXEL 3500 operating at $\lambda = 514.5$ nm, a thermostated cell holder, a detection system and a 90-channel multibit multiple-τ correlator that calculates the homodyne autocorrelation function $G^{(2)}(\tau)$. All experiments were done at a scattering angle of 90°. The laser power was adjusted to 1 W. The protein solution was injected through a 20 nm pore size filter (Protein Solutions Limited, UK) into a 100 μl flow-through micro cell (Hellma, FRG). Using the program CONTIN [14,15], the translational diffusion coefficient D was calculated from the autocorrelation function. The Stokes' radius R_s was determined from the Stokes-Einstein-relation: $R_s = kT(6\pi\eta_0 D)^{-1}$, where k is Boltzmann's constant, T the temperature in Kelvin and η_0 stands for the solvent viscosity.

2.4. CD measurements

CD measurements were performed on a J-720 spectrometer (JASCO, Japan) in 1 mm cells. The CD-spectrometer was calibrated with (+)-10-camphorsulfonic acid at 290.5 nm and 192.5 nm [16].

3. Results

First we measured the translational diffusion coefficient D with DLS for different protein concentrations in order to get the necessary D_0 for infinite dilution. This was done for various pH-values. Fig. 1 shows typical examples of the extrapolation of the translational diffusion coefficient D to zero protein concentration at 20°C. As expected strong virial effects appear for low pH values due to charged side chains. We find the Stokes' radius of RNase A under native conditions at 20°C and pH 7 to be $R_s = (1.90 \pm 0.04)$ nm.

Table 1
Influence of DTT on the Stokes' radius R_s of RNase A in 10 mM sodium cacodylate buffer, pH 7.5

pH	Addition (mM DTT)	R_s (nm)
7	0	1.90 ± 0.04
7.5	10	1.89 ± 0.04
7.5	100	1.90 ± 0.04

We also studied the effect of DTT on folded and unfolded RNase A in order to demonstrate the results of a faulty reduction process. In our experiments the addition of DTT alone does not give rise to an increase in R_s (Table 1). Our results are consistent with the fact that the disulfide bonds are buried in the protein core and are therefore not accessible to DTT. In order to correctly reduce RNase A previous unfolding is necessary (see section 2). It is also important to maintain reduced RNase A at a low pH in order to prevent reoxidation. In analogy to Sosnick and Trewhella [5] we performed temperature measurements on properly reduced RNase A (Table 2). We cannot confirm the results of reference [5] that reduced RNase A is further unfolded by temperature increase.

In order to finally determine the conformation state of reduced and GuHCl denatured RNase A, we studied the influence of GuHCl and DTT on the Stokes' radius of RNase A (Table 3) and on the corresponding CD spectra (Fig. 2). Specification of secondary structure content was not possible because GuHCl limits the measurements to wavelength of 215 nm. Reliable results require measurements down to at least 190 nm.

4. Discussion

Our DLS results given in Table 2 suggest that RNase A is already unfolded under reducing conditions at 20°C because no further increase of R_s with increasing temperature is indicated. In fact, we observe a slight decrease of the Stokes' radius when we increase the temperature from 20°C to 60°C. This is due to the temperature dependence of hydrophobic interactions. An increase in temperature enhances hydrophobic interactions which causes a slight compaction of RNase A.

Sosnick and Trewhella [5] chose reduction conditions in which they incubated RNase A in DTT for 1 h prior to data collection. Li et al. [17] studied the reduction of RNase A at 15°C time resolved by HPLC. After 33 h of incubation with DTT they still observed that RNase A was not fully reduced. Therefore we assume that Sosnick and Trewhella did measurements on RNase A with not all disulfide bonds destroyed.

We find that reduction and high concentrations of GuHCl lead to unfolding (Table 3). GuHCl denatured RNase A is less expanded than reduced RNase A. The Stokes' radius of reduced RNase A is greater with than without GuHCl suggesting that GuHCl is a good solvent for RNase A. For the radius of reduced-denatured RNase A we find $R_s = (3.14 \pm 0.06)$ nm. This is a 65% increase of the Stokes' radius from the native to the reduced-denatured state of RNase A. In the following, we will discuss this increase of R_s in relation to the dimension of other proteins unfolded by high concentrations of GuHCl. In a previous work [10], we have derived the scaling law $R_s = (0.28 \pm 0.03)\, N_A^{(0.50 \pm 0.03)}$ from Stokes' radii of 11 proteins lacking disulfide bonds or having reduced disulfide bonds. According to this scaling law, the calculated Stokes' radius of RNase A ($N_A = 124$) is 3.12 nm. The agreement with the experimentally determined $R_s = 3.14$ nm means that reduced-denatured RNase A has dimensions typical of highly unfolded proteins. If reduced-denatured RNase A were compact, all proteins with known hydrodynamic properties under highly unfolding conditions should then adopt a rather compactly folded conformation. In order to compare our hydrodynamic data to geometric data, a transformation via $R_G = \rho \cdot R_s$ is necessary. The factor ρ depends on the conformational state of the protein. The two extreme values are $\rho = 0.77$ and $\rho = 1.51$ where the former value is valid for a homogenous sphere and the latter value for an unperturbed random chain [6]. Geometric parameters of sufficiently high precision for proteins can only be delivered from X-ray or neutron scattering data, which are not attainable in general with X-ray scattering in the presence of high concentrations of GuHCl. A direct experimental determination of R_G for proteins unfolded by GuHCl has been achieved for only one of the proteins which were used to derive the scaling law mentioned above. Using small-angle neutron scattering on yeast phosphoglycerate kinase in 4 M GuHCl, Calmettes et al. [18] obtained $R_G = 7.8$ nm from the Guinier approximation and $R_G = 9.2$ nm from the Debye function for a random coil. We measured a Stokes' radius $R_s = 5.66$ nm. According to the relation $R_G = \rho \cdot R_s$ we get ρ-values of 1.38 and 1.62, respectively. The former value is less reliable, since in contrast to the Debye approximation, only a few data points were used for the Guinier approximation in that work. The latter value is very similar to our results obtained under conditions where the random chain conformation of the protein chain was characterized in detail by SAXS data. In that case we found $\rho = 1.55$ for acid-denatured apo-cytochrome c [19] and $\rho = 1.63$ for cold-denatured phosphoglycerate kinase [20]. Therefore we think that $\rho = 1.6$ is presently a rather good value for chemically-denatured proteins. Using this value we get $R_G = (5.0 \pm 0.1)$ nm for reduced-denatured RNase A. This value is larger than expected for a random coil at the θ-point according to Miller and Goebel [1]. Even if we assume ρ to be

Table 2
Temperature dependence of the Stokes' radius R_s of reduced RNase A

T (°C)	R_s (nm)
20	2.81 ± 0.06
40	2.75 ± 0.06
60	2.68 ± 0.05

Data for concentration $c = 2.53$ g/l, 50 mM MES buffer, pH 5.7 containing 1 mM EDTA; the dependence of R_s on c is neglectable (compare Fig. 1).

Table 3
Influence of DTT and GuHCl on the Stokes' radius R_s of RNase A

	No GuHCl	6 M GuHCl
Not reduced	(1.90 ± 0.04) nm[a]	(2.60 ± 0.05) nm[c]
Reduced	(2.91 ± 0.06) nm[b]	(3.14 ± 0.06) nm[d]

[a] 10 mM sodium acetate buffer, pH 4; c(RNase A) → 0
[b] 10 mM sodium acetate buffer, pH 4; c(RNase A) → 0
[c] 50 mM MES buffer, pH 5.7 containing 1 mM EDTA and 6 M GuHCl; c(RNase A) = 2.9 g/l*
[d] 50 mM MES buffer, pH 6.5 containing 1 mM EDTA and 6 M GuHCl; c(RNase A) = 2.9 g/l*
*The dependence on concentration is for these conditions neglectable.

1.38, the smallest determined value, we find R_G = (4.3 ± 0.1) nm. On the other hand, from R_G = 2.41 ± 0.1 nm determined for reduced-denatured RNase A by Sosnick and Trewhella [5] and our R_s = (3.14 ± 0.06) nm one gets ρ = 0.77. From this one would conclude that reduced-denatured RNase A is a compact sphere. This is very unlikely for the following reason. Our CD-spectrum is typical for a protein which contains no secondary structure. The ellipticity at 222 nm which is used to predict the amount of helicity is close to zero. If reduced-denatured RNase A were still globular it would be expected that its CD-spectrum would show considerable secondary structure. This is not observed. Moreover, for native RNase A R_G = 1.50 nm [5] and R_s = (1.90 ± 0.03) nm [this work] we get ρ = 0.79. This is a reasonable result for a globular protein which means that R_G = 2.41 nm and ρ = 0.77 are very unlikely for reduced-denatured RNase A. We cannot exclude the presence of a small amount of local secondary structure in the N terminus of reduced-denatured RNase A as was observed by Haas et al. [11] and Bierzynski and Baldwin [12].

The DLS results are consistent with the assumption that reduced-denatured RNase A is in a random-chain-like conformation for which essentially no secondary structure is expected. This is also supported by our CD-data.

Acknowledgements: This work was supported by grants from the Deutsche Forschungsgemeinschaft Da292/1–3, and Ga530/1–1. We would like to acknowledge G.D.'s grant from the Fonds der chemischen Industrie. We thank D. Otto for skillful technical assistance and G. Mohs (University of Arizona) for critically reading the manuscript.

References

[1] Miller, W.G. and Goebel, C.V. (1968) Biochemistry 7, 3925–3934.
[2] Tanford, C., Kawahara, K. and Lapanje, S. (1966) J. Biol. Chem. 241, 1921–1923.
[3] Greene Jr., R.F. and Pace, C.N. (1974) J. Biol. Chem. 249, 5388–5393.
[4] Aune, K.C., Salahuddin, A., Zarlengo, M.H. and Tanford, C. (1967) J. Biol. Chem. 242, 4486–4489.
[5] Sosnick, T.R. and Trewhella, J. (1992) Biochemistry 31, 8329–8335.
[6] Tanford, C. (1961) Physical chemistry of macromolecules, Chapter 6, John Wiley and Sons, Inc., New York.
[7] Flory, P.J. (1953) Principles of polymer chemistry, Chapter 14, Cornell University Press, Ithaca, New York.
[8] Bohdanecký, M. and Petrus, V. (1991) Int. J. Biol. Macromol. 13, 231–234.
[9] Flory, P.J. (1969) Statistical Mechanics of Chain Molecules, John Wiley and Sons, Inc., New York.
[10] Damaschun, G., Gast, K., Müller-Frohne, M. and Zirwer, D. (1995) Biopolymers, in press.
[11] Haas, E., McWherter, C.A. and Scheraga, H.A. (1988) Biopolymers 27, 1–21.
[12] Bierzynski, A. and Baldwin, R.L. (1982) J. Mol. Biol. 162, 173–186.
[13] Gast, K., Damaschun, G., Misselwitz, R. and Zirwer, D. (1992) Eur. Biophys. J. 21, 357–362.
[14] Provencher, S.W. (1982) Comp. Phys. Commun. 27, 213–227.
[15] Provencher, S.W. (1982) Comp. Phys. Commun. 27, 229–242.
[16] Johnson Jr., W.C. (1990) Proteins: Struct., Funct., Genet. 7, 205–214.
[17] Li., Y.-J., Rothwarf, D.M. and Scheraga, H.A. (1995) Nature Struct. Biol. 2, 489–494.
[18] Calmettes, P., Durand, D., Smith, J.C., Desmadril, M., Minard, P. and Douillard, R. (1993) J. Physique IV 3, 253–256.
[19] Damaschun, G., Damaschun, H., Gast, K., Gernat, C. and Zirwer, D. (1991) Biochim. Biophys. Acta 1078, 289–295.
[20] Damaschun, G., Damaschun, H., Gast, K., Misselwitz, R., Müller, J.J., Pfeil, W. and Zirwer, D. (1993) Biochemistry 32, 7739–7746.