Ribonuclease T1 has different dimensions in the thermally and chemically denatured states: a dynamic light scattering study

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Abstract Ribonuclease T1 can be unfolded and refolded without forming noticeable amounts of aggregates allowing to characterise the dimensions of a protein in different denatured states in terms of the Stokes radius $R_s$. Upon thermal unfolding $R_s$ increases from 1.74 nm at 20°C to 2.14 nm at 60°C. By contrast, $R_s = 2.40$ nm was obtained at 5.3 M guanidine hydrochloride (GuHCl) and 20°C. Heating from 20°C to 70°C in the presence of 5.3 M GuHCl led to a 5% decrease in $R_s$.

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1. Introduction

Our knowledge of the dimensions of proteins in unfolded and partially folded states is rather incomplete up to now. Only a few data exist as compared to numerous studies of the secondary structure of proteins in non-native states. However, a detailed knowledge of both secondary structure and compactness is necessary to understand the role of the starting conformation and the folding conditions for the rate and the yield of protein folding. In particular, the early steps of protein folding are only poorly understood. The question of whether local or global conformational changes are the dominant events has been intensively discussed [1-3]. Recently, the role of initiation sites of protein folding has again attracted much attention [4-6]. Further central questions are: how is the collapse of the polypeptide chain coupled with secondary structure formation upon folding [2] and what is the amount and the role of persistent structure in unfolded states [7]? Therefore, it is necessary to characterise the conformational properties of proteins in different unfolded states in more detail. A particular subject, which will be considered here are investigations of the dimensions of a protein in different unfolded states. Only a few reliable data of this kind exist [8,9], probably because of the experimental difficulties discussed below. One has to take into consideration that unlike studies of the native structure measured quantities of unfolded proteins are averages over an ensemble of conformations. The distance distribution function of the atomic structure is the best description of the physical dimensions of an unfolded protein. It is attainable by sophisticated X-ray and neutron scattering techniques [10]. The hydrodynamic Stokes radius, $R_h$, is a less direct measure of the physical dimensions. $R_h$ can be measured readily by dynamic light scattering (DLS). Size measurements of proteins in unfolded states are often hampered by the occurrence of aggregates upon unfolding. This is particularly true for thermal unfolding. In this respect, ribonuclease T1 (RNase T1) from Aspergillus oryzae is well suited for such investigations because it can be reversibly unfolded and refolded by heat and high concentrations of guanidine hydrochloride (GuHCl) without forming noticeable amounts of aggregates. RNase T1 comprises 104 amino acids and contains two disulfide bridges. The three-dimensional structure of the folded protein is well known from X-ray crystallographic [11,12] and NMR [13] studies. The energetics and kinetics of its unfolding and refolding have been intensively studied with the use of spectroscopic and calorimetric methods [14-21]. Nothing is known about the dimensions of the denatured protein in solution. As a first step in characterising the dimensions of RNase T1 in solution, we focus our attention on comparative studies of the Stokes radii of RNase T1 in the native, thermally unfolded, and GuHCl-unfolded states with its disulfide bonds intact. It would be interesting to obtain corresponding data for RNase T1 when one or both of its disulfide bonds are broken. Such investigations are the subject of future work.

2. Materials and methods

2.1. Materials

RNase T1 was obtained from Escherichia coli cells transformed with a plasmid carrying the chemically synthesised gene, which was cloned and expressed in E. coli [22] and purified as described elsewhere [23]. The lyophilized protein was dissolved directly in the respective solvents. Investigations of the native form and the thermal unfolding transition were performed in 10 mM sodium cacodylate buffer, pH 7 containing 1 mM EDTA. For the measurements of $R_h$ in the chemically denatured state the buffer was supplemented with 5.3 M GuHCl. Ultrapure GuHCl was obtained from ICN Biomedicals, Inc. The molarity was controlled refractometrically [17]. The protein concentrations were determined spectrophotometrically using an absorbance of $A(1$ cm, 0.1%$) = 1.9 at 277 nm.

2.2. DLS measurements

The laboratory-built DLS apparatus was described elsewhere [24]. 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-t correlator that calculates the homodyne autocorrelation function $G^2(t)$. All experiments were conducted at 90° scattering angle and 1 W laser power. The protein solutions

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Abbreviations: DLS, dynamic light scattering; RNase T1, ribonuclease T1; GuHCl, guanidine hydrochloride; CD, circular dichroism

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were injected through 20 nm pore size filters (Protein solutions Ltd., UK) into 100 μl flow-through micro cells (Helma, Germany). The translational diffusion coefficient \( D \) was calculated from the autocorrelation function using the program CONTIN [25]. The Stokes radius was obtained from the Stokes-Einstein-equation \( R_s = kT/6\pi\eta_0D \), where \( k \) is Boltzmann’s constant, \( T \) the temperature (in K) and \( \eta_0 \) the solvent viscosity. Solvent viscosities, \( \eta_0 \), were determined using an Ubbelohde type viscometer, ViscoBoy 2 (Lauda, Germany) and a digital density meter, DMA 58 (Anton Paar, KG, Austria).

3. Results and discussion

Fig. 1 shows the changes in the apparent Stokes radius, \( R_{s,\text{app}} \), on thermal unfolding at 3 concentrations, 0.8, 1.9, and 3.0 g/l, respectively. The time interval between measurements at adjacent temperatures was about 1 h, thus ensuring steady-state conditions during unfolding. Even in the case of the highest concentration, the Stokes radius after subsequent heating and cooling agreed with that before heating within the experimental error, which is less than 2%. This points to reversibility with respect to the dimensions. The vertical displacement of the individual curves is caused by a considerable concentration dependence of \( R_s \) at pH 7 and no additional salt. It is evident from Fig. 1 that the thermal transition is essentially complete at temperatures at and above 60°C. The continuous lines in Fig. 1 were obtained by non-linear least-squares fits of the data to the equation \( R(T) = (a+bT+(c+dT)\varepsilon)/(1+\varepsilon) \). \( a, b \) and \( c, d \) are parameters used to fit \( R(T) \) within the pre- and post-melting region, respectively. \( \varepsilon \) represents the expression \( \exp(-\Delta H_m/(1+\varepsilon)) \).

The Stokes radii of RNase Tl in the presence of 5.3 M GuHCl, measured at different temperatures for a protein concentration of 2.2 g/l, are shown in Fig. 3. The concentration dependence of \( D \) or \( R_s \), respectively, is very weak in the presence of high concentrations of GuHCl [26]. Therefore, the values in Fig. 2 practically coincide with that at \( c = 0 \). \( R_s \) is 2.40 ± 0.04 nm at 20°C and in the presence of 5.3 M GuHCl, where unfolding is complete. The decrease in \( R_s \) with increasing temperature will be discussed below.

On thermal unfolding the Stokes radius of RNase Tl increases by a factor \( f = R_{s,\text{mol}}/R_{s,\text{nat}} = 1.24 \). This is a considerably smaller expansion than that caused by unfolding by GuHCl at 20°C, where our data yield \( f = 1.38 \). Similar data have been published only for two proteins, lysozyme and ribonuclease A (RNase A). The increase in \( R_s \) on thermal unfolding is even smaller for these proteins. For lysozyme Nicoli and Benedek [27] obtained \( f = 1.18 \). We measured \( f = 1.16 \) in the case of RNase A (unpublished results). This is obviously due to the presence of disulfide bonds in both lysozyme and RNase A, which consist of 129 and 124 amino acids, respectively. In the case of GuHCl induced unfolding, the Stokes radius of lysozyme increased by a factor \( f = 1.45 \) [28], while \( f = 1.37 \) was determined for RNase A [29]. A further increase in \( R_s \) is observed after breaking the disulfide bonds.
For reduced and GuHCl-denatured RNase A, we found $R_s = 3.14$ nm resulting in $f=1.65$ [29]. It should be emphasized that in the case of RNase A the reduction of disulfide bridges alone led to unfolding at 20°C, resulting in an increase in $R_s$ from 1.90 nm to 2.91 nm. This radius decreased by 5%, when the temperature was raised to 60°C.

An increase in the radius of gyration, $R_g$, by a factor of 1.29 on thermal unfolding has been reported for RNase A with intact disulfide bridges [30]. The increase in $R_g$ and the changes in the distance distribution function on thermal unfolding of RNase T1 are described in detail in a subsequent paper (Damaschun et al., manuscript in preparation).

Now we will return to the observed temperature dependence of the Stokes radius of GuHCl denatured RNase T1 (Fig. 3). $R_s$ decreases by 5% from 2.40 nm at 20°C to 2.28 nm at 70°C. This decrease in $R_s$ is obviously a characteristic of proteins in the random coil conformation. A similar decrease in the intrinsic viscosities of some proteins unfolded by GuHCl was first observed and discussed by Ahmad and Salahuddin [31] and later by Privalov et al. [32]. The compaction of the polypeptide chain reflected by both the Stokes radius or the intrinsic viscosity was explained so far by facilitated backbone rotations in randomly coiled proteins [31] and/or by an increase in the hydrophobic forces [32] with increasing temperature. Desolvation of GuHCl bound to the polypeptide chain is probably not an important factor because a similar decrease in $R_g$ was observed for acid denatured apo-cytochrome c [26]. Our data further imply that there is some kind of convergence of the dimension in solutions in the absence and presence of high concentrations of GuHCl at high temperatures. Denaturants such as GuHCl interact preferentially with the surface of the polypeptide chain, particularly with hydrophobic side chains. Accordingly, in terms of polymer physics [33] a highly concentrated solution of GuHCl is a good solvent for the polypeptide chain leading to expansion of the random coil. On the other hand, in heat denaturation, thermal motion leads to a disordered conformation, but hydrophobic clusters within the otherwise unfolded chain may still persist. In other words, buffer without GuHCl is a poor solvent for the polypeptide chain also above the thermal melting transition. This could be the reason for the smaller Stokes radius obtained for thermally denatured as compared to GuHCl-denatured RNase T1.

Furthermore, it would be interesting to estimate the dimensions of RNase T1 lacking one or both of its disulfide bonds. RNase T1 is unfolded even at room temperature when the disulfide bonds are broken, but refolding to a native-like conformation can be induced by adding NaCl [34,35]. Measurements of the dimensions will be performed in future provided that the absence of protein aggregation is also maintained under these conditions.

While the observed changes in $R_s$ give a general idea of differences in the dimensions in different unfolded states, the question remains open whether the polypeptide chain adopts the conformation of a random coil with two cross-links or still comprises a considerable amount of native-like secondary structure. This question has partly been answered, albeit not unequivocally thus far, through spectroscopic methods. Fabian et al. [36] have studied the temperature-induced denaturation of RNase T1 by Fourier transform infrared spectroscopy. According to their data, temperature-denatured RNase T1 is predominantly, but not completely, in a random coil conformation. They exclude any residual $\alpha$-helical and $\beta$-sheet structure, but their spectra hint at some residual turn-like structures. This is in apparent contradiction to the results of circular dichroism (CD) measurements [14], from which the existence of some $\alpha$-helical and $\beta$-sheet structure could be derived. However, the observed CD effects need not necessarily be due to authentic secondary structure, but rather may result from a dynamic random structure having on average $\varphi$-$\psi$ angles similar to those observed for a static structure. CD cannot distinguish between dynamic and static structures [37]. Further investigations using scattering and spectroscopic methods are needed in order to obtain a more detailed description of the conformation of RNase T1 in different denatured states.

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