Dynamics of ribonuclease A and ribonuclease S: Computational and experimental studies

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Abstract

RNase S is a complex consisting of two proteolytic fragments of RNase A: the S peptide (residues 1–20) and S protein (residues 21–124). RNase S and RNase A have very similar X-ray structures and enzymatic activities. Previous experiments have shown increased rates of hydrogen exchange and greater sensitivity to tryptic cleavage for RNase S relative to RNase A. It has therefore been asserted that the RNase S complex is considerably more dynamically flexible than RNase A. In the present study we examine the differences in the dynamics of RNase S and RNase A computationally, by MD simulations, and experimentally, using trypsin cleavage as a probe of dynamics. The fluctuations around the average solution structure during the simulation were analyzed by measuring the RMS deviation in coordinates. No significant differences between RNase S and RNase A dynamics were observed in the simulations. We were able to account for the apparent discrepancy between simulation and experiment by a simple model. According to this model, the experimentally observed differences in dynamics can be quantitatively explained by the small amounts of free S peptide and S protein that are present in equilibrium with the RNase S complex. Thus, folded RNase A and the RNase S complex have identical dynamic behavior, despite the presence of a break in polypeptide chain between residues 20 and 21 in the latter molecule. This is in contrast to what has been widely believed for over 30 years about this important fragment complementation system.

Keywords: dynamics; MD simulation; ribonuclease A; ribonuclease S; tryptic cleavage

Fragment complementation systems are two or more fragments of a protein that can be reconstituted to form a complex with similar structure and activity to that of the native, uncleaved protein. Fragment complementation systems are a powerful tool in the study of protein folding and stability (Taniuchi et al., 1986). RNase S is one of the most well studied fragment complementation systems. It consists of S peptide (residues 1–20) and S protein (residues 21–124), two proteolytic fragments of the 124 amino acid protein RNase A. These fragments are obtained by cleavage of the peptide bond between Ala 20 and Ser 21 of RNase A by subtilisin. The two fragments can be reconstituted to give a noncovalently bound, active S peptide:S protein complex, RNase S (Richards & Vith-

ayathil, 1959). Residues 16–20 of the S peptide are not involved in binding of S peptide to S protein. The refined three-dimensional structures of RNase A and RNase S are now available to a high resolution (Wlodawer et al., 1988; Kim et al., 1992). The two proteins have very similar structures (Fig. 1) and enzymatic activity, but exhibit significant differences in their dynamics and stability (Richards & Wyckoff, 1971; Blackburn & Moore, 1982).

RNase S has been used as a model system to study the thermodynamics of protein folding. Because it is a bimolecular system, the thermodynamics of association can be measured very accurately using titration calorimetry (Connelly et al., 1990; Wiseman et al., 1989). Protein folding reactions involve a large decrease in heat capacity (Privalov & Gill, 1988). Understanding the origins of this change at the molecular level is an important fundamental problem. The decrease is currently thought to result principally from the release of ordered water molecules around hydrophobic groups in the folding process and has been correlated to the amount of nonpolar area buried when folded (Livingstone et al., 1991; Gomez et al., 1995). RNase A and RNase S have quite different heat capacities of folding/binding despite their identical folded structures. In addition, recent measurements of heat capacities of binding of various S peptide analogs to S protein clearly demonstrate that factors other than burial of nonpolar surface area can

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Abbreviations: $\Delta(X)$, (value of property 'X' in RNase A simulation) – (value of property 'X' in RNase S simulation); ASA, accessible surface area; CaBP, calcium binding protein; MD, molecular dynamics simulation; $\langle MD \rangle$, MD average structure; PMSF, phenylmethyl sulfonyl fluoride; RNase A, bovine pancreatic ribonuclease A; RNase S, product of limit digestion of RNase A with subtilisin; S peptide, the smaller (21 residues) fragment of RNase S; S protein, the larger (104 residues) fragment of RNase S.

Fig. 1. Plot of the C_{α} trace of the crystal structure of RNase S (continuous line) superimposed on the crystal structure of RNase A (dashed line). The structures were superimposed using the program INSIGHT II. Residues 24–124 were used in the superimposition procedure.

contribute significantly to the observed heat capacity of binding (Varadarajan et al., 1992). Differences in the dynamic properties of structurally similar molecules have been previously suggested as a possible explanation for these differences in heat capacity (Sturtevant, 1977; Varadarajan et al., 1992). We therefore attempt to characterize the dynamics of RNase S and RNase A. Although RNase S and RNase A have identical crystal structures (Kim et al., 1992), it is believed that RNase S shows much larger fluctuations around its time-averaged structure in solution. The evidence for this is as follows. At room temperature RNase S is readily cleaved by trypsin while RNase A is indefinitely stable (Allende & Richards, 1962). Tritium and deuterium exchange studies of RNase S (Rosa & Richards, 1981; Haris et al., 1986) also indicate that rates of exchange of amide protons from RNase S are much higher than those of RNase A (Woodward & Rosenberg, 1971; Englander, 1963). Kim et al. (1992) have made detailed comparisons of the various structures of RNase A and RNase S obtained by X-ray crystallography to investigate the possible role that structural differences may have played in the observed differences in dynamics and susceptibility to acid- and temperature-induced denaturation between the two proteins. Their comparison does not seem to suggest any reasons for the observed differences. We have therefore carried out 100 ps MD simulations of RNase A and RNase S at 300 K and 330 K in solution to investigate the possible origins of the above mentioned differences in the dynamics of RNase A and RNase S. One of the main advantages of MD simulations is the availability of information at an atomic resolution as a function of time (Brooks & Karplus, 1989; Brunne et al., 1993). We have also performed tryptic digestion studies in order to experimentally examine possible differences in the dynamics of RNase A and RNase S. Because fragment complementation systems are being extensively used as models for protein folding (Ruiz-Sanz et al., 1995) as well as in studies of protein structure and stability (Varadarajan & Richards, 1992; Varadarajan et al., 1992), it is important to understand the nature and origins of differences in the properties of the uncleaved protein and the fragment complex.

Results and discussion

MD simulations at 300 K

Although many biologically relevant motions in proteins occur on timescales of milliseconds to seconds, MD simulations of protein molecules in water are feasible for timescales of a few hundred picoseconds. The primary objective of our simulations was to characterize fluctuations around the time-averaged structures of RNase S and RNase A. Instead of performing one long simulation, we performed two 100-ps simulations for each molecule at temperatures of 300 and 330 K. In this section we report the results for the 300 K simulations. The average temperature and potential energy of RNase A and RNase S during the simulation are 304.05 K and 303.25 K, $-33,339.82 \text{ kcal mol}^{-1}$ and $-33,439.40 \text{ kcal mol}^{-1}$, respectively. The average RMS deviation (RMSD) of the coordinates of the structures obtained from the simulation from the energy minimized crystal structures of RNase A and RNase S are 0.98 Å and 0.75 Å for the main chain and 1.335 Å and 1.189 Å for the side chains, respectively. The MD trajectories of these parameters are shown in Figure 2. Although there is a slow drift in the potential energy during the simulation and the potential energy has not reached a final equilibrium value, the structure and fluctuations appear to have stabilized to a near equilibrium state. The RMSD of the coordinates of the backbone and side chain of RNase A and

Fig. 2. Molecular dynamics trajectories for the 300 K simulation of potential energy, temperature, RMSDs of the main chain (MC) and the side chains (SC) from the minimized starting structure. The panels on the left are those of RNase A, while the panels on the right are of RNase S.

RNase S from their minimized structures are comparable, with RNase S having a marginally smaller value. In Table 1, we have tabulated the average RMSD in coordinates from the (MD) structure, the average accessible surface area, and the number of waters that visit a given amino acid residue for nonpolar, polar, and charged residues for both the main chain and side chain atoms. The results of the simulations are analyzed in further detail below.

RMSDs

Backbone. We have computed an MD average structure and calculated the RMSD of the coordinates from this structure by residue over the simulation period. We now describe the dynamics of RNase A and RNase S and highlight their differences in terms of these RMSDs.

Shown in Figures 3A and 3B are the plots of the residue-averaged RMSD from the (MD) structure for the backbone atoms. The RMSDs are larger at the chain termini and in some of the loop regions. This is to be expected because these regions are less constrained than other regions of the protein and hence will show greater mobility. In RNase A (Fig. 3A), the regions consisting of residues 3–13, residues 24–34, and residues 103–112 have an RMSD of approximately 0.38 Å. This is significantly lower than the RMSD for other regions of the protein. The loop region connecting the S peptide and S protein, consisting of residues 15–23 has the highest RMSD and is the most mobile loop in RNase A. Subtilisin cleaves RNase A at the peptide bond between Ala 20 and Ser 21, which is a part of this loop. The two loop regions consisting of residues 66–70 and residues 86–90 also show a large RMSD. A glycine residue in the middle of each loop (G68 and G88) is responsible for the flexibility of these loops. The other glycine residue, G112, also has a large RMSD in both RNase A and RNase S. In Figure 3C, the difference RMSD between RNase A and RNase S, $\Delta(\text{RMSD})$, is shown along the length of the polypeptide chain with the residues 1–23 removed from the superposition procedure. In S peptide, residues 3–13 show more fluctuations in RNase S than in RNase A. This observation can be explained by the fact that in RNase S, S peptide is bound to S protein solely by noncovalent interactions, whereas in RNase A, there is also a covalent linkage. However, the increased mobility of the S peptide does not seem to affect the motion of the residues in S protein that interact with the S peptide. In S protein, residue Arg 39, residues 111–113, and residues in the 66–70 loop show more mobility in RNase S. This is in agreement with the observations of Kim et al. (1992), who on comparison of different crystal structures of

Fig. 3. Residue average RMSD of main chain atoms from the (MD) structure of the 300 K simulation. **A:** RNase A; **B:** RNase S; **C:** $\Delta(\text{RMSD})$. Residues 16–20 are absent in RNase S.

RNase A and RNase S found that the largest differences in these structures lie in the loop regions. Most secondary structural elements in RNase A show more fluctuation than in RNase S. For example, the fluctuations of residues 24–34 are higher in RNase A than in RNase S despite the fact that this helix is close to the N terminus of the S protein of RNase S. Large movements of the flexible loop consisting of residues 16–23, which precedes the helix 24–34 in RNase A, may have affected the dynamics of this helix, resulting in a large RMSD value. This loop is not covalently connected to the helix in RNase S. Kim et al. (1992) have suggested that structural differences in the loop region 34–38 could be responsible for the differences of RNase A and RNase S in behavior toward trypsin cleavage. In our simulations we do not see significant differences in the dynamics of this region.

Shown in Figures 4A and 4B are C_{α} traces of structures extracted at intervals of 5 ps from the RNase A and RNase S simulations, respectively. In each case the structures have been superimposed with the (MD) structure. From Figures 4A and 4B, it is clear that the backbone fluctuations in RNase A are similar but somewhat larger in magnitude than the fluctuations in RNase S.

Side chains. The coordinate RMSDs from the (MD) structure of the side chains of RNase A and RNase S are shown in Figures 5A and 5B. The overall RMSD of the side chains is approximately 0.64 Å, and is higher than the backbone RMSD for both RNase A and RNase S. The $\Delta(\text{RMSD})$ obtained after superimposing the S protein region of the structures is shown in Figure 5C. As in the case for the main chain RMSDs, the two proteins also have very

Table 1. RMSD and the average values of ASA, and number of visits over the simulation for main chain (MC) and side chain (SC) atoms

RNase	Residue Type	RMSD (Å)		ASA (Å) ²		Visits	
		MC	SC	MC	SC	MC	SC
A	Nonpolar	0.43	0.64	5.6	18.1	8.6	9.9
S	Nonpolar	0.39	0.56	3.8	18.0	6.2	10.7
A	Polar	0.50	0.66	15.0	50.1	15.0	15.4
S	Polar	0.44	0.59	13.4	51.2	11.2	18.1
A	Charged	0.45	0.59	10.8	78.6	10.2	24.0
S	Charged	0.46	0.64	10.2	73.4	7.8	24.9

Fig. 4. Superposition of the C_{α} traces of structures extracted every 5 ps from the 300 K simulation on the $\langle MD \rangle$ structure. **A:** RNase A; **B:** RNase S.

similar side chain RMSDs. Minor differences are seen in the side chain fluctuations of hydrophilic residues such as Asn 67, Gln 69, and Asn 113, which are often correlated with the fluctuations in relevant side chain hydrogen bonds.

Solvent accessible area

The solvent accessible surface areas of RNase A and RNase S were calculated for the minimized structures, as well as for structures extracted from the simulation at 5 ps intervals. The average

and RMSDs of the areas were calculated. The fluctuations in accessible surface area are extremely small, ranging from 0.4 to 3%. Furthermore, we did not observe any significant differences in the average accessible areas of RNase A and RNase S (Table 1).

Correlation analysis

Analysis of coordinate RMSDs did not reveal any significant differences between RNase A and RNase S dynamics. Therefore we also calculated the differences in the covariance matrices between RNase A and RNase S. We did not find any appreciable differences in the covariance, indicating the absence of differences in correlated motions between RNase A and RNase S during the simulation.

Correlation between visits of water and hydrogen exchange rates

Tritium and deuterium exchange experiments (Rosa & Richards, 1981; Haris et al., 1986) indicate faster amide exchange rates in RNase S than in RNase A. We calculated the number of visits that water molecules make to the hydration shells of each atom in the protein in RNase S and RNase A during the simulation and attempted to correlate this with experimentally observed exchange rates. As with other properties, the number of visits is quite similar for RNase A and RNase S, with the main chain of RNase A having a slightly higher number of visits than the main chain of RNase S, in contrast to the experimental results. The number of visits depends on the residue type as well as on the shape of the protein surface. We observe that the number of visits is lowest for non-polar residues and highest for polar residues. This implies a longer residence time for water molecules around nonpolar residues. This is consistent with the fact that water is more ordered around hydrophobic residues than around polar or charged residues.

The exchange rates of amide protons of RNase A have been studied by NMR spectroscopy (Santoro et al., 1993). All the main chain amide protons of RNase A have been divided into three classes: (1) protons that exchange with a rate constant of less than $1.5 \times 10^{-4} \text{ min}^{-1}$, (2) protons that have an exchange rate constant greater than $1.5 \times 10^{-2} \text{ min}^{-1}$, and (3) protons that have an exchange rate constant that lies between these two limits. We find that there is a definite correlation between the number of visits to a given residue observed in the simulation and the exchange class

Fig. 5. Residue average RMSD of side chain atoms from the $\langle MD \rangle$ structure of the 300 K simulation. **A:** RNase A; **B:** RNase S; **C:** $\Delta(\text{RMSD})$.

of that residue determined by NMR (Fig. 6). As we proceed from the set of residues that have 5 visits to the set of residues with 35 visits, we observe that the fraction of fast exchanging protons increases while the fraction of slow exchanging protons decreases. All amino acid residues that have greater than 30 visits belong solely to the class of residues that have the highest amide proton exchange rate constants. The timescales of simulation are very different from those of the experiment. However, the excellent correlation between the results of the MD simulation and experimental exchange studies for RNase A indicate that if there are genuine differences in the exchange behavior of folded RNase A and complexed RNase S, these should be visible in the simulation.

Simulations at 330 K

It is well known that the potential energy surface of a protein in the native state consists of large number of local minima (Elber & Karplus, 1987). MD simulations of time scales of 100 ps sample only a fraction of the conformation space available to the protein. The obvious way to enhance the conformational sampling is to perform long and exhaustive simulations. The longest known simulations to date of proteins solvated by a large number of explicit water molecules have been of the order of 1 nanosecond. Several biologically important motions occur on timescales of 1 nanosecond to milliseconds. Thus, any long MD simulation is always short when compared with biological timescales (Dagget & Levitt, 1993). One method of enhancing sampling of conformational space, given the limitations in computer resources, is to perform simulations at elevated temperatures. High temperature simulations have been previously used to study the unfolding of globular proteins (Mark & Gunsteren, 1992; Dagget & Levitt, 1993; Vijayakumar et al., 1993). We have performed simulations of RNase A and RNase S at two different temperatures, 300 K and 330 K, each of 100 ps length. Figure 7 shows the trajectories of the temperature, potential

Fig. 6. Correlation between the number of visits to a given residue and its amide proton exchange class. The fraction of protons in the three exchange classes are drawn on the Y axis as a histogram [exchange rate $< 1.5 \times 10^{-4} \text{ min}^{-1}$ (■), exchange rate $> 1.5 \times 10^{-2} \text{ min}^{-1}$ (■), exchange rates that are between $1.5 \times 10^{-4} \text{ min}^{-1}$ and $1.5 \times 10^{-2} \text{ min}^{-1}$ (■)].

Fig. 7. Molecular dynamics trajectories for the 330 K simulation of potential energy, temperature, RMSDs of the main chain (MC) and the side chains (SC) from the minimized starting structure. The panels on the left are those of RNase A, while the panels on the right are of RNase S.

energy, and RMSDs from the crystal structure of the main chain and the side chains for RNase A and RNase S. The temperature and the potential energy remain stable with small fluctuations. As is evident from the figure, the RMSDs of the main chain of RNase A and RNase S are comparable (1.1 Å), while the RMSD of the side chains of RNase A is greater than that of RNase S by about 0.5 Å. The fluctuations of the main chains and the side chains around the (MD) structure of RNase A and RNase S also show small differences (Fig. 8).

Thus, the simulations of 100 ps each of RNase A and RNase S at temperatures of 300 K and 330 K indicate similar dynamic behavior around their time-averaged structures. An exhaustive analysis of data from the simulations revealed only minor differences in the dynamics between RNase A and RNase S. These differences were largely confined to loop regions and to the chain termini. RNase S contains two additional chain termini relative to RNase A. Of these two, only the C terminus of the S peptide helix exhibits significantly greater dynamic flexibility. Although some other residues also exhibit altered behavior in the RNase S simulations, it is unclear whether these differences are due to minor differences in the starting structures for the two simulations. The simulations strongly suggest that, apart from these minor differences, both RNase A and RNase S in solution exhibit similar fluctuations in the area of their respective time-averaged structures. This is in apparent contradiction with previous proteolysis (Allende & Richards, 1962) and hydrogen exchange (Haris et al., 1986; Rosa & Richards, 1981) data. This apparent difference could

Fig. 8. Residue average Δ RMSDs of atoms from the (MD) structure of the 330 K simulation. MC, main chain; SC, side chain.

simply be due to the fact that simulation times were not long enough to observe the differences. However, we note that there was a reasonable correlation of the simulation results for RNase A with experimental measurements of hydrogen exchange rates even though the two studies examined quite different timescales. In addition, there were no substantial differences in fluctuations between the two proteins even in the simulations performed at a higher temperature of 330 K. We therefore chose to examine an alternate explanation for the apparent differences between simulation and experiment.

Experimental studies

Trypsin digestion studies of RNase A and RNase S

Because it was observed from the MD simulations that RNase A and RNase S exhibited similar dynamic behavior, we reexamined the experimental data that had suggested that RNase S should exhibit greater dynamic flexibility than RNase A. There are two different probes that have been used for monitoring the dynamics of RNase A and RNase S, hydrogen exchange (Haris et al., 1986; Rosa & Richards, 1981; Woodward & Rosenberg, 1971) and trypsin digestion (Allende & Richards, 1962). Allende and Richards (1962) have demonstrated that RNase S can be digested by trypsin at room temperature. RNase A can only be digested under extreme conditions, for example, at a temperature of 60°C (Ooi et al., 1963). We reexamined the experimental evidence to see if the resistance to cleavage of RNase A could be explained by factors other than differences in dynamic flexibility between RNase A and RNase S.

A fundamental difference between RNase A and RNase S is that RNase A is a unimolecular system while RNase S is bimolecular.

Thus the fraction of RNase A present in the folded state should be independent of the total (folded + unfolded) RNase A concentration. In contrast, because folded RNase S is always in equilibrium with S peptide and S protein, the fraction of folded RNase S will be a function of the total (folded RNase S + S protein) RNase S concentration (Schreier & Baldwin, 1976). Because the dissociation constant of RNase S is approximately 10 nM, it has generally been assumed that the small amount of S protein present in most experiments could be neglected (Allende & Richards, 1962; Rosa & Richards, 1981). However, a more detailed analysis reveals that this is not correct. At the time the original exchange experiments were performed (Schreier & Baldwin, 1976; Rosa & Richards, 1981) the rate constants for association and dissociation of S protein from S peptide were not known and there was only limited information on the thermodynamics of binding of S peptide to S protein. The rate constants for association were determined subsequently by Labhardt et al. (1983), and a complete thermodynamic characterization of the binding as a function of temperature was made recently by titration calorimetry (Varadarajan et al., 1992). We have reanalyzed the proteolysis experiments in light of this fresh data and have also performed trypsin digestion studies of RNase A and RNase S as a function of protein concentration. Because RNase S is a bimolecular system, the tryptic digestion of RNase S can proceed through either of the two pathways shown below.

In the first pathway, proteolysis occurs in the folded undissociated complex. In the second pathway, RNase S dissociates into S peptide and S protein. Each of these molecules is subsequently proteolyzed by trypsin. The extent of the possible contribution of the second pathway can be analyzed as follows. k_1 and k_{-1} are the rate constants for dissociation and association of S peptide and S protein, respectively. The value of k_{-1} as measured by stopped flow studies is $1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Labhardt et al., 1983) at pH 6.8, 30 °C. Under similar conditions the measured dissociation constant (K_d) for RNase S is 10^{-7} M (Hearn et al., 1971; Varadarajan et al., 1992). Hence k_1 is estimated to be approximately $1.2 \times 10^{-1} \text{ s}^{-1}$. The proteolysis experiments of Allende and Richards (1962) were performed at pH 8.0, 25 °C, at an RNase S concentration of 0.043 mM and a trypsin concentration of 0.43 μM . The dissociation constant for S peptide:S protein binding at 25 °C is similar to that at 30 °C (Varadarajan et al., 1992). Hence at a protein concentration of 0.043 mM, the concentrations of complexed RNase S and S protein will be 0.041 and 0.002 mM, respectively. The concentration of S peptide will be equal to that of S protein. The rate of dissociation of RNase S will be $k_1[\text{RNase S}] = 4.9 \times 10^{-6} \text{ M s}^{-1}$. If the system is at equilibrium, this will be equal to the rate of association of S peptide and S protein. The rate of cleavage of S protein will be given by

$$k_{cat}[\text{trypsin}][\text{S protein}]/(K_m + [\text{S protein}]). \quad (2)$$

k_{cat} for trypsin is of the order of 5 s^{-1} for peptide and protein substrates (Pace & Barrett, 1984; Noda et al., 1994). The K_m of trypsin for small peptides and unfolded proteins is of the order of 0.01–0.1 mM (Pace & Barrett, 1984; Noda et al., 1994). The few

estimates of K_m values of trypsin with folded proteins that are readily digested by trypsin are in the range of 0.1–0.2 mM (Labeyrie, 1956; Ram & Maurer, 1957). It is well known that folded proteins are typically digested much more slowly than unfolded ones (Coligan et al., 1995). We assume that the slower rates of digestion of folded proteins must result primarily from an increase in K_m of trypsin for a folded protein relative to an unfolded or partially folded protein. This assumption has been shown to be correct in trypsin digestion studies of native and denatured lactoglobulin (Labeyrie, 1956). The K_m of trypsin for S protein is expected to be higher than the 0.1 mM value observed for unfolded proteins and peptides. At an enzyme concentration of 0.43 μ M and an S protein concentration of 0.002 mM, the rate of cleavage of S protein estimated by Equation 2 will be smaller than $4.3 \times 10^{-8} \text{ M s}^{-1}$. This is considerably slower than the rate of formation of S protein from RNase S. Since $K_m \gg [\text{S protein}]$, the rate of cleavage of RNase S by the second pathway is thus given by

$$\begin{aligned} -d/dt[\text{RNase S}] &= k_{cat}[\text{trypsin}][\text{S protein}]/K_m \\ &= k_{cat}[\text{trypsin}]\sqrt{K_d[\text{RNase S}]/K_m}. \end{aligned} \quad (3)$$

Substituting the values of k_{cat} , K_d , and $[\text{trypsin}]$ given above we obtain

$$-d/dt[\text{RNase S}] = 6.8 \times 10^{-10} \sqrt{[\text{RNase S}]/K_m}. \quad (4)$$

Integration of equation (4) gives

$$\sqrt{[\text{RNase S}]}(t=0) - \sqrt{[\text{RNase S}]}(t) = 3.4 \times 10^{-10} t/K_m. \quad (5)$$

At a starting RNase S concentration of 0.041 mM, the experimentally observed time for 50% digestion is 90 minutes (Allende & Richards, 1962). According to Equation (5), a K_m value of 1 mM for S protein would be consistent with this digestion time. This is similar to the value of K_m of S protein estimated from tryptic digestion studies of free S protein (see below). This strongly suggests that the second pathway described above can contribute significantly during proteolysis of RNase S even under conditions where the S protein concentration is a small fraction of the RNase S concentration. If digestion was occurring through the first pathway, the rate of digestion of RNase S would be given by

$$-d/dt[\text{RNase S}] = k_{cat}[\text{trypsin}][\text{RNase S}]/(K_m + [\text{RNase S}]). \quad (6)$$

No estimates are available for the K_m of trypsin for folded RNase S. However, because digestion of RNase S occurs more slowly than that of S protein, the K_m is expected to be higher than the K_m for S protein. Hence the $[\text{RNase S}]$ term in the denominator of Equation (6) can be neglected in comparison with K_m and the equation becomes first order with respect to RNase S. Because the K_m of trypsin for folded RNase S is not known, the time taken for 50% digestion cannot be estimated. However, in contrast to the second pathway, it will be independent of the initial RNase S concentration at protein concentrations less than 1 mM. In order to confirm whether proteolysis through the second pathway was significant, we performed tryptic digestions of RNase A and RNase S as a function of total RNase S concentration, at a trypsin concentration of 0.01 mg/mL (0.43 μ M). The range of RNase S concentrations explored was 1–100 μ M. This is well below the expected K_m of trypsin for RNase S.

It was found that the digestion of RNase S by trypsin at 20 °C was strongly concentration dependent (Fig. 9A,B). Digestion of 0.001 mM RNase S was complete within 90 minutes, while for 0.1 mM RNase S, a much smaller fraction of the protein, was digested in the same time. In contrast, RNase A was completely resistant to proteolytic digestion at all protein concentrations studied at 20 °C (Fig. 9B). This is the expected result if indeed digestion of RNase S is occurring through free S protein. Under these conditions for starting RNase S concentrations of 1, 5, and 20 μ M, the estimated times (using Equation 5 and $K_m = 1 \text{ mM}$) for 50% digestion via the second pathway are approximately 15, 30, and 60 minutes, respectively. These calculated half lives qualitatively agree with the trend seen in the gels (Fig. 9A,B). We have also performed digestions of S protein at 20 °C (Fig. 9C). S-protein at 20 °C also exhibited a half life that increased with increasing protein concentration. It is known (Richards & Wyckoff, 1971; Goldberg & Baldwin, personal communication) that unlike

Fig. 9. Concentration dependent tryptic digestion. Digestion conditions: pH = 8, 10 mM Hepes, 1 mM CaCl_2 . Equal amounts of protein (1 μ g) loaded on a SDS PAGE gel. RNase S migrates as S protein due to dissociation of S20 peptide under denaturing gel conditions. Marker protein (M) is a mixture of RNase A and S in panels A, B, C, and E, and is undigested CaBP for panel D. Numbers above the brackets denote time of digestion in minutes. **A,B:** RNase S and RNase A digestion at 20 °C (0.01 mg/mL trypsin). Each set of four wells (marked by a bracket) consists of digests performed at substrate concentrations of 0.1, 0.02, 0.005, and 0.001 mM, respectively. RNase S, panel A and lanes 1–4 and 5–8, panel B. RNase A, lanes 10–13, panel B. **C:** S protein (0.01 mg/mL trypsin) digestion at 20 °C. The concentrations and amounts of protein in a set of four wells are as in **A**. **D:** CaBP digestion at 20 °C (0.01 mg/mL trypsin). Loaded 0.3 μ g CaBP. Concentrations of CaBP in each set as in **A**. **E:** Digestion of RNase A+S mixtures at 20 °C (0.01 mg/mL trypsin) keeping the total RNase A+S concentration constant at 0.1 mM. One μ g of RNase S is loaded in each well. Each set of three wells (marked by a bracket) consists of digests performed at 0.1, 0.02, and 0.005 mM RNase S, respectively, with substrate concentration made up to 0.100 mM in each case by adding RNase A.

RNase S and RNase A, S protein shows a tendency to aggregate at concentrations above 10 μ M. It is therefore likely that this aggregation is responsible for the dependence of the half life of S protein on the initial S protein concentration. The digestion kinetics of S protein at the lowest concentration used in these experiments (1 μ M) can be used to obtain an approximate value for the K_m of trypsin for S protein of about 2 mM. This is similar to the value of 1 mM used in the analysis of the RNase S digestion studies. The discrepancy between the values may arise because of partial aggregation of the S protein even at concentrations of 1 μ M.

As a control, we performed tryptic digests with another protein, CaBP, which was easily digested under similar conditions, and found that the half life for cleavage over an identical substrate concentration range was relatively independent of substrate concentration, as would be expected for a first order reaction (Fig. 9D). In order to clearly demonstrate that the concentration dependent digestion of RNase S is a specific effect that is not simply due to an increase in total protein concentration, we performed digestion experiments of RNase S in the presence of RNase A. In these experiments the sum of the RNase A and RNase S concentration was kept constant while varying the RNase S concentration. These studies were performed at two different total protein concentrations of 0.02 mM and 0.100 mM. In each case similar results were obtained. The digestion at 0.100 mM total protein concentration is shown in Figure 9E. The rates of tryptic cleavage of RNase S are unaffected by the presence of RNase A (compare gels in Fig. 9A, 9B, and 9E). This demonstrates that the concentration dependence of the half life observed for RNase S is a specific effect that depends solely on the initial concentration of RNase S. The agreement between the measured cleavage rates at different RNase S concentrations and the calculated rates for the S protein mediated cleavage pathway in Equation 1 strongly suggests that this is the major cleavage pathway and that at room temperature folded RNase S, like RNase A, is insensitive to trypsin.

Another possible explanation for the apparent dependence of half life on protein concentration for S protein and RNase S would be if K_m for these two proteins was much smaller than the protein concentrations used in the experiments. Equation (6) would then become zero order with respect to the substrate. Since the rate of digestion would be constant and independent of the initial concentration, the time taken for complete digestion would increase linearly with the initial substrate concentration. The k_{cat} of trypsin for these substrates would then have to be several orders of magnitude lower than the previously observed value of 5 s⁻¹ (Pace & Barret, 1984; Noda et al., 1994) in order to explain the observed times of digestion. The K_m would also have to be substantially lower than the known K_m values of trypsin for peptides and denatured and native proteins (Pace & Barret, 1984; Labeyrie, 1956). Furthermore, it would have to be assumed that the differences in proteolytic sensitivity between RNase A, RNase S, and S protein are due primarily to difference in k_{cat} rather than K_m . None of these assumptions appears reasonable and we therefore discount this latter explanation.

Hydrogen exchange studies

We next reanalyzed the hydrogen exchange experiments of Haris et al. (1986) in the following way. As with proteolysis, tritium exchange in RNase S can occur through two distinct pathways.

Here the asterisk (*) indicates labeled protein. k_1 and k_{-1} are the rate constants for dissociation and association, respectively, of S peptide from S protein. k_{ex} is the average rate constant for exchange from free S peptide and free S protein and $k_{ex\#}$ is the average rate constant for exchange from the RNase S complex. An approximate estimate of $k_{ex} = 5 \times 10^{-3} \text{ s}^{-1}$ can be obtained by averaging the measured values of k_{ex} for 20 amide protons in S protein at pH 7.0, 15 °C (Tables 1 and 2 of the tritium exchange data of Rosa & Richards, 1981). The value of k_1 (see above) was estimated to be $1.2 \times 10^{-1} \text{ s}^{-1}$. The dissociation constant (K_d) for RNase S is estimated by titration calorimetry to be $1.45 \times 10^{-8} \text{ M}$ at 15 °C (Varadarajan et al., 1992). Using these values it is straightforward to show that, as in the case of proteolysis, the second step involving exchange is much slower than the first step of peptide dissociation and will be equal to the rate of formation of RNase S. We therefore obtain

$$\sqrt{[\text{RNase S}^*]}(t=0) - \sqrt{[\text{RNase S}^*]}(t) = 3.01 \times 10^{-7} t. \quad (8)$$

Haris et al. (1986) have used FTIR to monitor deuterium exchange of RNase A and RNase S under identical conditions using a protein concentration of 3.65 mM. RNase S was observed to have a measurably higher rate of exchange. At these concentrations, although only 0.3% of the RNase S is dissociated, the time taken for complete exchange via S protein, calculated according to Equation (8), will be 2.3 days. This is in reasonable agreement with the experimental observation of Haris et al. (1986) that exchange is complete within 8 days for RNase S but not for RNase A. Thus exchange through S protein can account for the apparent differences in hydrogen exchange observed between RNase A and RNase S. In order to confirm this we have initiated tritium exchange studies as a function of total protein concentration.

RNase S is the most well characterized fragment complementation system and has been the subject of a very large number of biophysical studies since its discovery over 35 years ago. RNase S is produced from RNase A by cleavage at a single peptide bond. The cleavage occurs within a highly solvent accessible loop that has few interactions with the remainder of the protein and is therefore not expected to appreciably destabilize the molecule. In addition, RNase S and RNase A have very similar structures and activities. The exact origins of the experimentally observed increase in dynamic flexibility in RNase S relative to RNase A were not understood. However, it seemed intuitively reasonable that cleavage of the polypeptide chain should be accompanied by such an effect.

We have studied the dynamic behavior of RNase A and RNase S computationally, through MD simulations, and experimentally through proteolysis studies. The MD simulations do not show any significant differences in dynamic behavior between the two proteins. An advantage of the MD simulations is that they allow folded RNase A and complexed RNase S to be compared under identical conditions and in the absence of unfolded protein or dissociated complex. This is not possible to do experimentally. Both our and earlier experimental observations can be quantita-

tively explained by a simple model in which the apparent experimentally observed differences in RNase A and RNase S dynamics are due to the small amounts of free S peptide and S protein that are in equilibrium with RNase S. This analysis, together with the MD simulation data, demonstrate that folded RNase A and the RNase S complex have similar dynamic behavior despite the presence of a break in the polypeptide chain in the latter molecule.

In addition to providing a satisfactory answer to the long standing question of the origins of differences in RNase A and RNase S dynamics, these studies help in the understanding of the relationship between a fragment complementation system and the corresponding intact protein. It is an obvious but often forgotten fact that the stability and dynamic behavior of a fragment complex will be concentration dependent, while those of a monomeric protein will not. These studies also emphasize that, this difference aside, fragment complementation systems can be excellent model systems for studying protein folding, stability, and dynamics.

Materials and methods

Materials

Trypsin (TPCK treated), RNase A (Type XII A), and RNase S (Type XII S) were obtained from Sigma Chemical Co. RNase A and RNase S were purified in a Pharmacia FPLC system using a 1-ml RESOURCE S column. RNase A and RNase S elute at 100 mM NaCl at pH 6, 25 mM phosphate buffer. The proteins were extensively dialyzed against 10 mM Hepes at pH 8 to remove NaCl and phosphate. S protein was prepared from RNase S by reverse phase HPLC in acetonitrile-water using a Vydac C-18 preparative column. CaBP, a calcium binding protein (MW = 14.7 kD) from *Entamoeba histolytica*, was purified as described (Prasad et al., 1993).

Methods

Cleavage of ribonuclease with trypsin

Trypsin was stored at -20°C as a stock of 1 mg/mL in 20 mM CaCl_2 , 20 mM sodium acetate, pH 5 (Flannery et al., 1989) up to a period of two weeks. Conditions for proteolysis of RNase S, RNase A, S protein, and CaBP were as follows. All digests were performed in 10 mM Hepes pH 8.0, 1 mM calcium chloride at a temperature of 20°C . The final concentration of CaCl_2 for CaBP was 5 mM. Trypsin was added to final concentrations ranging from 10–50 $\mu\text{g/mL}$. Digests were performed for times ranging from 2–180 minutes. At intervals, aliquots were taken out and digestion stopped by adding SDS-PAGE loading buffer containing 2 mM PMSF. Samples were immediately frozen in liquid nitrogen and later boiled to irreversibly inactivate trypsin. Subsequently, equal amounts of total protein were loaded and analyzed by SDS-PAGE. Protein bands were visualized by Silver staining. RNase A, RNase S, and S Protein concentrations were estimated using an extinction coefficient of $9.56\text{ mM}^{-1}\text{ cm}^{-1}$ at 280 nm (Connelly et al., 1990). CaBP concentrations were estimated based on an extinction coefficient of $5.1\text{ mM}^{-1}\text{ cm}^{-1}$ at 280 nm.

Simulation protocol

The simulations of RNase A and RNase S were performed with the AMBER 4.0 suite of computer programs (Pearlman et al., 1991) using the protocol described below. The starting models for the two proteins were obtained from X-ray crystallography. For the

simulations of RNase A, we used the $1.26\text{ \AA}$ structure of the phosphate free RNase A (Wlodawer et al., 1988). RNase S was simulated using the $1.65\text{ \AA}$ structure of the truncated 15-residue S peptide:S protein complex (Kim et al., 1992). The sulphate ion present in the crystal structure of RNase S was not included in the simulation. The crystallographically determined water molecules in the structures of RNase A as well as RNase S were also removed from the starting model for the simulations and the protein was solvated with water as follows.

Using the BOX option in the EDIT module of AMBER, we placed the coordinates of the two proteins at the center of a pre-equilibrated box of TIP3P Monte Carlo water molecules. Those waters that made bad van der Waals contact with any protein atom were discarded, while remaining waters within a distance of $5\text{ \AA}$ from any protein atom along the X, Y, and Z axes were retained. This procedure resulted in the protein being solvated by 2,393 waters in the case of RNase A and 2,389 waters in the case of RNase S. Each water molecule consists of three atoms. Our model for the simulations consists of 1,219 protein atoms for RNase A and 1,187 protein atoms for RNase S. The total number of atoms in each simulation is thus 8,398 atoms for RNase A and 8,372 atoms for RNase S. The protein was modelled using the united atom force field of AMBER, with all the polar hydrogens explicitly included. The amino acid residues Arg and Lys were protonated, while each of the Glu and Asp residues carried a net negative charge. All the histidine residues except His 12 were protonated at Ne^2 and $\text{N}\delta^1$, while His 12 was protonated only at Ne^2 .

We then performed 100 steps of energy minimization using the steepest descent method with harmonic restraints of $5,000\text{ kcal mol}^{-1}\text{ \AA}^{-2}$ on the protein coordinates. The MD simulation consisted of a 10 ps equilibration, followed by a 90 ps production run. The equilibration was started by assigning velocities drawn from a Maxwellian distribution at 100 K to all the atoms. The equations of motion were integrated for a period of 2 ps using a 1 femtosecond time step during which the temperature was maintained by coupling the system to a heat bath with a time constant of 0.1 ps. The harmonic constraints on the protein coordinates were reduced to $2,500\text{ kcal mol}^{-1}\text{ \AA}^{-2}$. In the next step of the equilibration, the temperature was raised to 200 K, with a subsequent reduction in the value of the harmonic restraints on the protein coordinates to $1,250\text{ kcal mol}^{-1}\text{ \AA}^{-2}$. The last 6 ps of equilibration were done at 300 K with no restraints on the protein. This was followed by a 90 ps NVE simulation. The simulations at 330 K consisted of a 20 ps equilibration period of which the first 10 ps had the same protocol as that of the simulations at 300 K. The remaining 10 ps of equilibration was done at 330 K by coupling the system to a heat bath with a time constant of 0.1 ps. During the simulations all bond lengths were held to their equilibrium values using the SHAKE (Ryckaert et al., 1977) algorithm. The nonbonded interactions were computed using a residue-based list with a cut off of $12\text{ \AA}$. The list was updated every ten time steps. A dielectric constant of $\epsilon = 1$ was used. The coordinates were saved every 0.1 ps, resulting in 900 sets of coordinates. The coordinate set obtained by taking the average of these coordinate sets is the MD average ((MD)) structure. The computations were done on IBM RS6000 340 workstation. Five ps of a typical run required 22 hours of CPU time.

Analysis

The analysis of the MD coordinate trajectories of the two simulations was performed, and the results were compared as follows.

RMSDs and ASA. The RMSDs between the structures obtained from the simulation and the minimized structures were calculated after superimposing each of these structures on the minimized structure using an algorithm suggested by Kearsley (1989). The RMSD was calculated separately for the backbone and the side chain atoms. The atomic fluctuations in RNase A and RNase S were studied by computing the RMSD of the structures generated from the simulation from the (MD) structure. The RMSD values were averaged over the backbone and side chain atoms for each residue. The difference in RMSD from the average structure between RNase A and RNase S was also calculated. ASAs were calculated using the Lee and Richards (1971) algorithm with a probe radius of 1.4 Å.

Number of visits. The number of waters that solvate the protein was calculated by finding the number of waters that are within a sphere of radius 4 Å of any protein atom. A cut off of 4 Å was used to include waters that are within the first hydration shell of all the protein atoms. Protein structures were extracted from the simulation at intervals of 5 ps. For each structure in turn, we calculated the number of waters within 4 Å of any atom of a given amino acid residue and stored their identity. In the subsequent structure we checked the identity of the waters that solvated the residue. The number of new water molecules that entered the first hydration shell by displacing the old waters or by additionally entering the shell is the number of visits to that residue (Brunner et al., 1993). This procedure was repeated for all the structures and the value of the number of visits updated. The number of visits to the main chain and side chain were then tabulated (Table 1).

Correlation. We studied collective motions of protein atoms during the simulation by constructing a $N \times N$ covariance matrix (Ichiye & Karplus, 1991) of the displacements of the C_α atoms from the (MD) structure. The covariance matrix was calculated for every structure from the RNase A and RNase S simulations and averaged over these structures to generate the average covariance matrix. The differences in the average covariance matrices of RNase A and RNase S were examined.

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