Ribonuclease A Revisited: Infrared Spectroscopic Evidence for Lack of Native-like Secondary Structures in the Thermally Denatured State†

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ABSTRACT: To address a number of conflicting reports in the literature, we undertook an infrared spectroscopic study to test for the presence of native-like secondary structures in thermally denatured ribonuclease A. Ribonuclease A does not aggregate at high temperatures, and the infrared spectrum shows a completely featureless amide I band contour. Using ^{13}C-labeled urea, we were also able to obtain the infrared spectrum of the chemically denatured protein, which is practically identical with that of the heat-denatured protein. To the best of our knowledge, this is the first study that uses ^{13}C-labeled urea as a chemical denaturant which circumvents the problem encountered with the strong absorption of urea in the conformation-sensitive amide I region of proteins; it opens up the possibility of investigating protein folding/unfolding processes in the presence of high concentrations of chemical denaturants. From an analysis of the amide I region of the infrared spectra of thermally and chemically denatured RNase A, it was concluded that heat-denatured ribonuclease A does not contain any significant amount of authentic hydrogen-bonded secondary structures. Furthermore, a comparison of the infrared spectra of ribonuclease A with those of ribonuclease T1 demonstrates that in spite of major differences between their native structures there are practically no differences between their heat-denatured states. This would not be expected if there were residual native-like secondary structures in the thermally denatured state of one or both of these proteins.

The continuing attention paid to the different denatured states of proteins (Dill & Shortle, 1991) is related to their involvment in at least three important phenomena: (i) protein folding and stability, (ii) transport across membranes, and (iii) proteolysis and protein turnover. However, it is intrinsically difficult to obtain detailed structural information on the denatured states of proteins; so far this information has been derived almost exclusively from studies of their macroscopic properties. It has become apparent in recent years that the denatured states of proteins are often not well described as random coils but that they may possess residual structure; in particular, there is now evidence that significant hydrophobic interactions may exist in the denatured state of many proteins (Shortle, 1993; Wüthrich, 1994).

One of the most extensively studied proteins, ribonuclease A (RNase A),[1] is a relatively small, monomeric protein. Small-angle X-ray scattering data have shown that thermally denatured RNase A undergoes only a 30% increase in its radius of gyration when its disulfide bonds remain intact (Sosnick & Trewhella, 1992). On the other hand, in the presence of the chemical denaturant guanidine hydrochloride (GuHCl), the protein unfolds into a much looser structure with increased overall dimensions, though smaller than that predicted for a random coil structure (Sosnick & Trewhella, 1992). Based on the fact that large signals in the far-UV region of the spectrum of heat-denatured RNase A were observed by CD, which disappeared by addition of GuHCl, it was concluded (Labhardt, 1982) that there is a significant amount of secondary structure in thermally denatured RNase A. In particular, it was suggested that the α-helix content in RNase A decreases during thermal denaturation to only about half of that found in the native protein. In contrast, calorimetric data (Privalov et al., 1989) indicate that the hydrophobic groups in thermally denatured RNase A are largely solvated, which argues against close contacts of these residues in the denatured protein. More recently (Robertson & Baldwin, 1991), hydrogen-exchange labeling in combination with NMR spectroscopy has shown that essentially all of the amide backbone protons in heat-denatured RNase A exchange at a rate very close to that predicted for a totally disordered polypeptide. The discrepancy between the NMR data on one side and the CD data on the other side raises the question whether large CD signals in the far-UV region are diagnostic for stable hydrogen-bonded structures, and therefore whether indeed there is residual secondary structure in RNase A after thermal denaturation.

In order to address this discrepancy, we compare herein infrared spectra of RNase A after thermal denaturation with those after chemical denaturation. The spectra were obtained under strictly comparative conditions. Notwithstanding certain limitations (Surewicz et al., 1993; Jackson & Mantsch, 1995), Fourier transform infrared spectroscopy has

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[1] Abbreviations: RNase A, ribonuclease A; RNase T1, ribonuclease T1.

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emerged as a valuable tool for following conformational changes in proteins (Casal et al., 1988; Schlereth & Mäntele, 1993; Fabian et al., 1993, 1994). So far, infrared studies on temperature-induced conformational changes in proteins were limited by the fact that most proteins showed irreversible intermolecular β-aggregation upon thermal denaturation. On the other hand, the common chemical denaturants GuHCl or urea, which must be used at high concentrations to ensure protein unfolding, themselves have strong infrared bands that interfere with the conformation-sensitive amide I bands of proteins. The protein we chose for this study, RNase A, does not aggregate, and its thermal unfolding is reversible. Furthermore, to circumvent the problem associated with the presence of high concentrations of the chemical denaturant, we have resorted to using ^{13}C-labeled urea.

MATERIALS AND METHODS

Ribonuclease A (type XII-A), urea, and ^{13}C-labeled urea were purchased from Sigma Chemical Co. (St. Louis). Infrared spectra were measured as solutions in D_2O in the presence or absence of ^{13}C-labeled urea. Deuterated [^{13}C]-urea was prepared by repeated lyophilization from [^{13}C]urea/D_2O. Typically, 2 mg of the lyophilized protein was dissolved in 40 μL of the corresponding solvent. Sample pH was adjusted with DCl or NaOD and is reported as measured, without correction for the deuterium isotope effect. Protein solutions were placed between a pair of CaF_2 windows separated by a path length of 45 μm for the thermal denaturation studies, or 6 μm for samples containing the chemical denaturant (8 M). Measurements were performed on a Digilab FTS-40A spectrometer equipped with a liquid nitrogen-cooled MCT detector and continuously purged with dry air. For each sample, 512 interferograms were co-added and Fourier-transformed to obtain a spectrum with a nominal resolution of 2 cm^{-1}. The sample temperature was controlled by means of a thermostated cell jacket. To obtain spectra at discrete temperatures, the protein solutions were heated in intervals of 5 °C. Spectra at these temperatures were recorded by equilibrating the sample for 10 min prior to data collection which itself took 6 min. Solvent spectra were recorded under identical conditions and subtracted from the spectra of the proteins in the relevant solvent. Extreme care was taken to keep the same purge level during the measurements. Minor spectral contributions from residual water vapor were eliminated using a set of water vapor spectra, as described earlier (Fabian et al., 1993). The final unsmoothed protein spectra with a signal-to-noise ratio greater than 10 000:1 were used for further analysis. Band-narrowing by Fourier self-deconvolution was performed as described previously (Mantsch et al., 1988) using a half-bandwidth of 15 cm^{-1} and a band-narrowing factor $k = 2.0$.

RESULTS

Thermal Denaturation of RNase A. Figure 1 shows three infrared spectra of RNase in D_2O (pH 5.5), at 20, 65, and 75 °C, recorded upon heating, and also a spectrum recorded after cooling the protein sample from 75 °C down to 20 °C. The measurements were initiated only after complete N^1H-N^2H exchange; therefore, the spectral changes observed as a function of temperature solely reflect structural changes due to the thermal unfolding of the protein. Complete N^1H-N^2H exchange was achieved by keeping the protein solutions

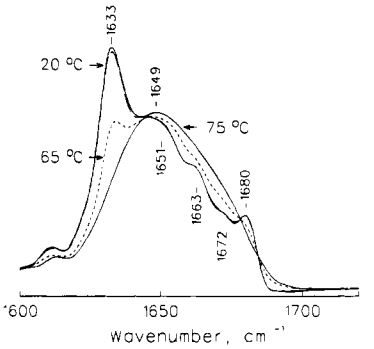

FIGURE 1: Infrared spectra of RNase A in D_2O at pH 5.5 at the indicated temperatures. All spectra are shown after Fourier self-deconvolution performed with the parameters given under Materials and Methods. Note that the spectra obtained at 20 °C before heating (solid line at 20 °C) and after cooling from 75 °C down to 20 °C (long dash-dotted line at 20 °C) are practically superimposable.

in D_2O for 30 min at a temperature a few degrees below the denaturation temperature (i.e., 60 °C). The individual amide I band components in the infrared spectrum of native RNase A were previously assigned (Haris et al., 1986; Olinger et al., 1986) to characteristic secondary structural elements: the major amide I component at 1633 cm^{-1} to β-strands, the bands at 1663 and 1672 cm^{-1} to turn structures, and bands in the range 1640−1652 cm^{-1} to irregular protein segments and α-helical structures. The band component at 1680 cm^{-1} is associated with turns and antiparallel β-sheets. The thermal unfolding of RNase A results in clear spectral changes, as illustrated in Figure 1 for three selected temperatures. The spectrum obtained after cooling the protein sample from 75 °C down to 20 °C is practically identical with that at 20 °C before heating. This also applies to the measurements performed at pH 2.1 and pH 7.4, indicating that the thermal unfolding of RNase A is reversible over the pH range 2.1−7.4, even at the relatively high protein concentrations used here. This experimental fact is important for the ensuing discussion. After complete thermal denaturation at 75 °C, the spectrum exhibits only a broad, nearly featureless amide I band contour centered at 1649 cm^{-1} and thus is quite different from the spectrum recorded at 20 °C. Practically identical amide I band contours were obtained from thermally unfolded RNase at pH 2.1 and also at pH 7.4. The infrared band at 1633 cm^{-1}, assigned to β-strands and the dominant feature in the spectrum of the native protein at 20 °C, is still present though less intense in the spectrum of RNase A at 65 °C. At temperatures above 70 °C, it dissappears as a discrete band, even when extensive resolution enhancement is applied. This clearly shows that the β-strands, which comprise almost half of the secondary structure of native RNase A, are absent in the thermally denatured state.

The infrared spectroscopic characterization and quantification of α-helical structures and, in particular, the correlation between temperature-induced spectral changes and changes in the α-helical content of proteins are less straightforward. While amide I bands between 1650 and 1658 cm^{-1} are generally considered to be characteristic of α-helical structures (Surewicz et al., 1993), for some proteins known to be largely α-helical, the major amide I component is shifted to wavenumbers below 1650 cm^{-1}. In such cases, the amide I band(s) of α-helical structures may overlap with those of irregular structures, making the unequivocal assignment of

amide I components to α-helical or irregular structures difficult, or even impossible. Three helical regions are known to exist in RNase A. The classical α-helical region comprises residues 3−13 (helix I), while helix II (residues 24−34) and helix III (residues 50−60) are distorted; part of helix III is actually a 3_{10} helix (Wlodawer et al., 1982; Borkakoti et al., 1982). The minor, though distinct, differences in the hydrogen-bonding pattern of the amide C=O groups in the three helices will impact on the frequencies of the individual amide I modes, which in turn leads to a wider distribution of the amide I band components. This could result in an infrared spectrum which has no single sharp amide I band component between 1640 and 1655 cm^{-1} that can be unequivocally correlated with a given α-helical structure, though the total helical content of RNase A is ~25%. This fact complicates the search for residual α-helical structures in thermally denatured RNase A. While the highly diagnostic infrared band at 1633 cm^{-1} is a sensitive probe of conformational changes in β-strands, an equally diagnostic band for α-helical structures is missing in the infrared spectrum of RNase A. The broad amide I band contour in the thermally denatured protein centered at 1649 cm^{-1} rather suggests a predominatly irregular structure. However, the presence of residual α-helical structures in the thermally denatured RNase A cannot be excluded on the basis of its infrared spectrum.

Chemical Denaturation of RNase A. For a comparison with the thermally denatured protein, we also obtained the infrared spectrum of chemically denatured RNase A, using a common chemical denaturant, urea. A direct comparison of the infrared spectra of the thermally and the chemically denatured state of a protein is important, since the latter is often considered to be close to the "random coil" state (Tanford, 1968). Potential differences between the infrared spectra of heat-denatured RNase A and chemically denatured RNase A should be indicative of conformational differences between the two unfolded states. In the absence of such conformational differences, practically identical spectra are expected. Obtaining good infrared spectra of proteins in the presence of the chemical denaturant, which must be used at high concentrations to ensure protein unfolding, is not simple and has not been very successful so far. First, infrared cells with short path lengths are required to prevent a saturation of the infrared detector by the absorption of the chemical denaturant. Second, the major infrared band of urea masks the weaker amide I bands. To circumvent this we have resorted to labeling urea with carbon-13 which causes the C=O band of urea to shift from 1618 cm^{-1} to 1562 cm^{-1} (in D_2O solution), leaving a clear window in the infrared spectrum for observing the protein amide I bands above 1600 cm^{-1}.

The combination of using infrared cells with a short path length and ^{13}C-labeled urea has allowed us to measure infrared spectra of RNase A in the presence of high concentrations of the chemical denaturant and still obtain useful information from the conformation-sensitive amide I band of the protein. A comparison of the amide I band contour of heat-denatured RNase A (solid trace in Figure 2) with that of the chemically denatured protein (broken trace in Figure 2) shows practically identical amide I band profiles, indicating that the different denaturing conditions lead to identical (or very similar) unfolded states of RNase A. Since chemical denaturation is generally used as the unfolded

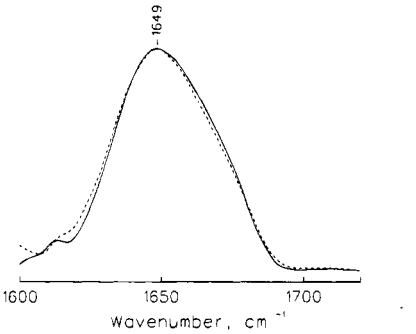

FIGURE 2: Infrared spectra (after Fourier self-deconvolution) of heat-denatured RNase A (65 °C) at pH 2.1 (solid line) and of chemically denatured RNase A in 8 M deuterated [^{13}C]urea, pH 3.8 (broken line).

reference state, it follows that there is practically no native-like secondary structure in the thermally unfolded RNase A either. The minor differences between the two amide I band contours in Figure 2 may reflect only slight, if any, differences in the distribution of the individual amide I modes in the predominantly irregular protein. The weak infrared absorbance at 1613 cm^{-1} is due to the amino acid side-chain absorption of tyrosine (Fabian et al., 1994). In this context, it is worthwhile to mention amide hydrogen exchange studies reported with another protein, lysozyme. The protection factor for exchange in the thermally and urea-denatured state of lysozyme, which was small and of almost identical magnitude, could be well approximated for both states by use of the random coil model parameters (Buck et al., 1994).

Comparison with RNase T1. Because the thermal unfolding of RNase A is reversible and since there are no indications for native-like secondary structures in its denatured state, we were encouraged to compâre the infrared spectrum of thermally denatured RNase A with that of heat-denatured RNase T1, another protein whose thermal unfolding is known to be reversible (Fabian et al., 1993). Interestingly, RNase T1 is yet another protein for which CD data have suggested a significant amount of secondary structure in its heat-denatured state (Oabatake et al., 1979). In contrast, recent infrared spectroscopic data indicate that the thermally unfolded state of RNase T1 is predominantly, though not completely, irregular. Residual, turnlike structures were suggested to exist in the heat-denatured state of RNase T1 with no residual β-structure (Fabian et al., 1993). Furthermore, certain point mutations were found to impact on the native structure of RNase T1 without affecting its thermally denatured state (Fabian et al., 1994). An inspection of the infrared spectra of native RNase A and RNase T1 (Figure 3A) reveals major differences in their secondary structure (a fact already well-known from X-ray analysis), whereas the spectra of the two heat-denatured proteins are pratically identical (Figure 3B). If there were residual native-like secondary structures in the heat-denatured state of one or both of these proteins, their infrared spectra after thermal denaturation should be different. Since this is obviously not the case, the infrared data argue against the existence of native-like structures in the thermally denatured state of these two proteins. It thus appears that in the unfolded state proteins with different amino acid sequences and different native structures have identical or very similar infrared spectra in the amide I region that are representative of the sum of all the individual amide I bonds. It then follows that within the limits of the information which can be derived

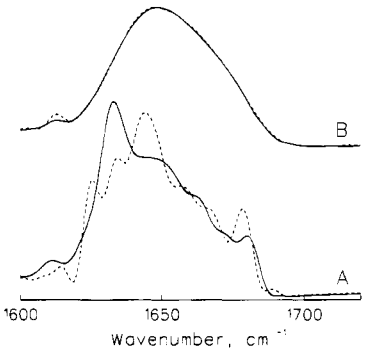

FIGURE 3: (A) Comparison of the infrared spectrum of native RNase A, pH 5.5 (solid line), with that of native RNase T1, pH 7.0 (broken line), at 20 °C after normalization using the spectra of the corresponding thermally unfolded proteins. (B) Comparison of the infrared spectrum of thermally denatured RNase A, pH 5.5 (solid line), with that of thermally denatured RNase T1, pH 7.0 (broken line), at 75 and 70 °C, respectively. All spectra are shown after Fourier self-deconvolution performed with the parameters given under Materials and Methods. The spectra of RNase T1 are from Fabian et al. (1994).

from the distribution of the peptide backbone C=O groups by infrared spectroscopy, the conformations assumed by thermally unfolded RNase A and RNase T1 do not depend on their amino acid sequences.

DISCUSSION

The infrared spectroscopic results presented herein represent a significant addition to the existing pool of data on the thermally denatured state of RNase A and also permit a more general discussion of the denatured states of proteins. The analysis of the amide I bands, which implies the lack of any native-like secondary structure in thermally denatured RNase A, is in excellent agreement with hydrogen-exchange studies by NMR (Robertson & Baldwin, 1991). It is important to note that the infrared measurements and the NMR NH-exchange experiments involve different approaches: the former a static one, the latter a dynamic one. The fact, that different probes which report on different aspects of the structure of the heat-denatured state of RNase A come to practically identical conclusions, cannot be underestimated. The main argument between the NMR and the CD data on RNase A revolves around the fact that the secondary structure determined by CD could be the result of fluctuating structures which would not give a high degree of hydrogen-exchange protection (Sosnick & Trewhella, 1992; Seshadri et al., 1994). However, such fluctuating structures with transient hydrogen bonding, if present, should also be detectable by infrared spectroscopy due to the sensitivity of the amide oxygen to H-bonding and the short time scale of the experiment; the present infrared data do not support the existence of such structures. It must be stated, however, that though the infrared data presented herein argue against the existence of authentic, native-like secondary structures in thermally unfolded RNase A, the presence of a broad ensemble of structural elements which, in particular, could resemble those of short α-helices with a slightly different hydrogen-bonding pattern cannot be totally excluded on the basis of the infrared spectra alone.

At variance with our results are two recent infrared studies (Sosnick & Trewhella, 1992; Seshadri et al., 1994). A slightly structured amide I band contour in the infrared spectrum of RNase A, measured at 66 °C, was taken as an indication for residual secondary structure in thermally denatured RNase A at pH 5.7 (Sosnick & Trewhella, 1992). In particular, a weak amide I band component at 1633 cm^{-1} was correlated with residual β-sheet structure. As discussed earlier, we also observed a weak amide I band component at 1633 cm^{-1} under similar experimental conditions, i.e., at pH 5.5 and 65 °C (see broken line in Figure 1). This band, however, disappeared after complete thermal denaturation above 70 °C. Thus it is safe to conclude that the infrared spectra discussed by Sosnick and Trewhella reflect the incomplete heat denaturation, rather than indicate the existence of residual secondary structure in thermally unfolded RNase A. In another study (Seshadri et al., 1994), a well structured amide I band in the infrared spectrum of RNase A at pH 2.0 and 65 °C was taken as evidence that the thermally denatured state of RNase A still possesses about half the helix and β-sheet content of the native state. This is at variance with our spectra measured under comparable experimental conditions, i.e., pH 2.1 and 65 °C (see solid line in Figure 2), which show only a broad, nearly featureless amide I band contour, practically identical with the amide I band contours obtained for heat-denatured RNase A at pH 5.5 and at pH 7.4. An incomplete unfolding of RNase A is less likely since the thermal unfolding of RNase A at pH 2 is complete at 65 °C. In our view, a more likely source of disparity is the low signal-to-noise ratio (about 500:1), compared to the very high signal-to-noise ratio in the present study (>10 000:1). A high signal-to-noise ratio and elimination of the sharp water vapor bands (often invisible by the naked eye) are critical prerequisites before applying resolution enhancement techniques such as differentiation or deconvolution; otherwise, the separation of overlapping bands may lead to artifacts in the resolution-enhanced spectra [see, e.g., Jackson and Mantsch (1995)].

To the best of our knowledge, this is the first comparison of infrared spectra from thermally and chemically denatured RNase A. The present data suggest that at the level of the secondary structure the unfolded states of RNase A show no differences. Thus, although the thermally denatured state of RNase A is more compact than its chemically denatured state(s) (Sosnick & Trewhella, 1992), this is most likely not the result of native-like regions of secondary structure in the heat-denatured state. The infrared data, however, do not exclude the presence of other, e.g., hydrophobic, interactions; hydrophobic clusters may exist even in a urea-denatured protein, such as recently shown for a domain of the 434-repressor protein (Neri et al., 1992). Interactions of this kind, if present in the denatured states of RNase A, might not affect the infrared spectrum and thus will not be detected by infrared spectroscopy. Calorimetric data, on the other hand, argue against close contacts of hydrophobic groups in denatured RNase A (Privalov et al., 1989).

Finally, the strategy introduced in this paper, which utilizes ^{13}C-labeled urea as a chemical denaturant, should find a wider range of applictions in future comparative studies of unfolded states of proteins, particularly so since the chemically denatured state of a protein is often used as the starting point in folding experiments from which, by dilution of the denaturant, the folding process from the denatured to the native state is initiated (Dobson et al., 1994). Furthermore, low concentrations of chemical denaturants are also used to generate stable intermediates of protein folding such as

"molten globules". With ^{13}C-labeled urea as a chemical denaturant, such conformational studies should now be possible by infrared spectroscopy as well.

REFERENCES

Borkakoti, N., Moss, D. S., & Palmer, R. A. (1982) *Acta Crystallogr., Sect. B B38*, 2210−2217.

Buck, M., Radford, S. E., & Dobson, C. M. (1994) *J. Mol. Biol. 237*, 247−254.

Casal, H. L., Köhler, U., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 957*, 11−20.

Dill, K. A., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795−825.

Dobson, C. M., Evans, P. A., & Radford, S. E. (1994) *Trends Biochem. Sci. 19*, 31−37.

Fabian, H., Schultz, C., Naumann, D., Landt, O., Hahn, U., & Saenger, W. (1993) *J. Mol. Biol. 232*, 967−981.

Fabian, H., Schultz, C., Backmann, J., Hahn, U., Saenger, W., Mantsch, H. H., & Naumann, D. (1994) *Biochemistry 33*, 10725−10730.

Haris, P., Lee, D. C., & Chapman, D. (1986) *Biochim. Biophys. Acta 874*, 255−265.

Jackson, M., & Mantsch, H. H. (1995) *Crit. Rev. Biochem. Mol. Biol. 30*, 95−120.

Labhardt, A. M. (1982) *J. Mol. Biol. 157*, 331−355.

Mantsch, H. H., Moffatt, D. J., & Casal, H. L. (1988) *J. Mol. Struct. 173*, 285−298.

Neri, D., Billeter, M., Wider, G., & Wüthrich, K. (1992) *Science 257*, 1559−1563.

Oabatake, M., Takahashi, S., & Ooi, T. (1979) *J. Biochem. (Tokyo) 86*, 55−63.

Olinger, J. M., Hill, D. M., Jakobsen, R. J., & Brody, R. S. (1986) *Biochim. Biophys. Acta 869*, 89−98.

Privalov, P. L., Tiktopoulo, E. I., Venyaminov, S. Yu., Griko, Y. V., Makhatadze, G. I., & Khechinashvilli, N. N. (1989) *J. Mol. Biol. 205*, 737−750.

Robertson, A. D., & Baldwin, R. L. (1991) *Biochemistry 30*, 9907−9914.

Schlereth, D. D., & Mäntele, W. (1993) *Biochemistry 32*, 1118−1126.

Seshadri, S., Oberg, K. A., & Fink, A. L. (1994) *Biochemistry 33*, 1351−1355.

Shortle, D. (1993) *Curr. Opin. Struct. Biol. 3*, 66−74.

Sosnick, T. R., & Trewhella, J. (1992) *Biochemistry 31*, 8329−8335.

Surewicz, W. K., Mantsch, H. H., & Chapman, D. (1993) *Biochemistry 32*, 389−394.

Tanford, C. (1968) *Adv. Protein Chem. 23*, 122−283.

Wlodawer, A., Bott, R., & Sjölin, L. (1982) *J. Biol. Chem. 257*, 1325−1332.

Wüthrich, K. (1994) *Curr. Opin. Struct. Biol. 4*, 93−99.

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