Structuring of Denatured Ribonuclease-A

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Summary Denatured aqueous ribonuclease-A is shown by high-field ^{13}C n.m.r. spectroscopy to be predominantly structured even at elevated temperatures and with its disulphide bonds chemically cleaved.

RIBONUCLEASE-A is commonly used as a paradigm of reversible protein unfolding. Although Roberts[1] and others[2,3] have noted that ribonuclease-A is not completely unfolded by heat alone, heat plus 8 M urea is considered to effect full unfolding on the basis of the ^{1}H n.m.r. spectra of the histidyl residues. We have investigated this further by a ^{13}C n.m.r. study at 100·6 MHz, at which frequency many of the carbon atoms in the protein give resolved resonances.

As well as noting that a few individual resonances are affected by preliminary unfolding below the main transition temperature for denaturation (to be published), we find that the resonances from the native protein are reversibly replaced as a group over the temperature interval 315 to 325 K by a new group of resonances, many of which clearly show a chemical shift heterogeneity inconsistent with that expected for a truly random-coil peptide.

For example, the upper spectrum in the Figure shows three separate resonances at δ_C *ca.* 11. From their shifts these must almost certainly arise from C-δ of the three isoleucine residues in the protein, for no other peptide resonance lies within 4 p.p.m. Each methyl group must be in a unique well-defined average environment in order to give a resolved, sharp resonance. Furthermore, this environment is probably not solvent-like, because the normal shift for this resonance in a peptide is δ 11·3—12·5 in water and 11·0 in dimethyl sulphoxide.[4] Similarly, three peaks are observed for C-γ of the three phenylalanine residues, at δ *ca.* 137.

The chemical shift heterogeneity of all identifiable groups of resonances from like carbon atoms in the denatured protein is typically 1 p.p.m., which is considerably less than that found in native proteins,[4] and simplifies the assignments. In general, hydrophobic residues such as isoleucine and phenylalanine, and including threonine, show well-resolved resonances, whereas hydrophilic residues such as glutamate, tyrosine, and histidine show single, slightly broad resonances analogous to those of lysine C-δ and C-ϵ in native proteins. Their linewidths are consistent with the sidechains sampling a variety of environments, and spending typically 0·01 s in each. Also, all the glutamate residues appear to have a similar pH-dependence. All these observations, together with previous T_1 measurements,[3] are consistent with the denatured protein having a relatively loosely bound, expanded structure held together by hydrophobic interactions. The expansion of molecular volume is presumably sufficient to account for the hydrodynamic behaviour[2] which is normally interpreted as that of a random coil. The general reduction in chemical shift heterogeneity may be explained by free rotation of all aromatic rings. We observed a similar spectrum for the protein at pH 1·2 and 6·0 and also in 8 M urea at 335 K, with many single carbon resonances still resolved, although broader.

In order to find out whether this structuring is merely due to the four S–S bridges we also obtained spectra where these had been cleaved either by dithiothreitol reduction[5] or by performic acid oxidation.[6] Surprisingly, the chemical shift heterogeneity is retained in both cases (see the Figure) even at elevated temperatures. The spectra show the expected changes in the cysteinyl resonances, and, following oxidation, in the methionine resonances at δ 15·2. They also differ from each other in detail, which shows that the chemical shift heterogeneity is not due to some hitherto unobserved effect of primary structure. But they all have the general features described above, with resolved resonances from hydrophobic residues and broad ones from hydrophilic.

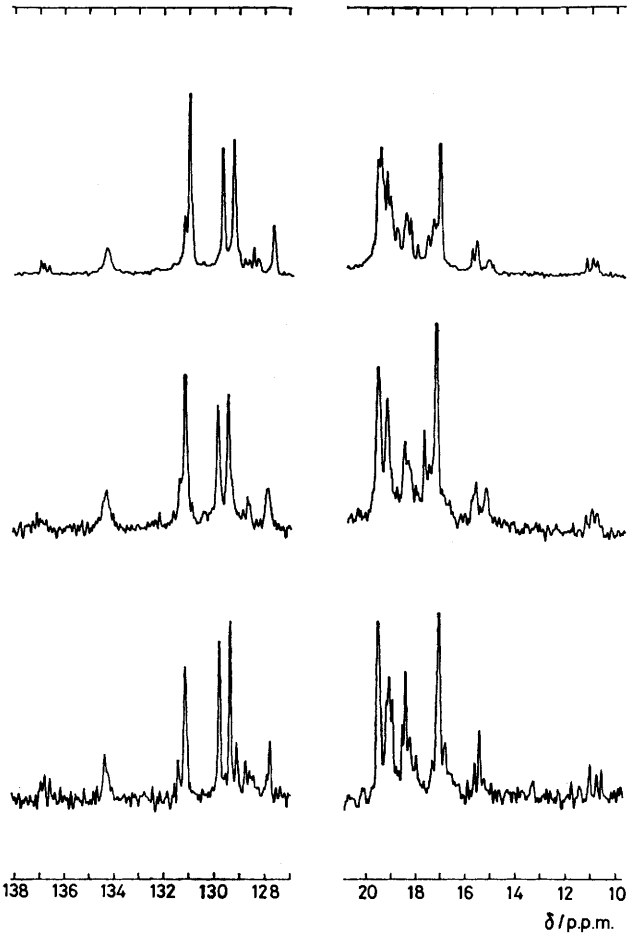

FIGURE. Portions of the 100·6 MHz ^{13}C n.m.r. spectrum of denatured ribonuclease-A. (Upper trace): Thermally denatured at 330 K, pH 3·3, 10 mM. The same spectrum was also obtained at 2 mM. (Middle trace): Reduced by dithiothreitol, 325 K, pH 3·3, 9·7 mM. (Lower trace): Oxidised by performic acid, 308 K, pH 4·4, 8 mM. A very similar spectrum was obtained at 325 K, but with some evidence for an additional aggregated component.

This suggests that a hydrophobically bound structure is likely to be a general feature of soluble denatured proteins, and calls into question any theoretical or kinetic study of protein folding made on the assumption that the denatured protein is a random coil.

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