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The use of osmolytes to facilitate protein NMR spectroscopy

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SUMMARY

A method of stabilizing folded proteins is described, which allows NMR studies under conditions where a protein would normally be unfolded. This enables stable proteins to be examined at elevated temperatures, or spectra recorded on samples that are insufficiently stable under normal conditions. Up to two molar perdeuterated glycine, a potent osmolyte, can be added to aqueous protein NMR samples without altering the folded three-dimensional structure or function of the protein. However, the stability of the folded form is dramatically increased. This is illustrated for the protein lysozyme at high temperature (348 K) where the structural integrity is destroyed in standard aqueous solution, but is retained in the osmolyte solution. We hope that the technique will be of value to those studying by NMR the structural biology of protein fragments and mutants, which are often of reduced stability compared with the original proteins.

At the present time, the chief methods for determining high-resolution three-dimensional (3D) protein structures are X-ray crystallography and NMR. Each technique presents a particular set of problems. For crystallography, the main difficulty is obtaining crystals that diffract to sufficient resolution; with NMR the major limitation is size, as most proteins are too large. To overcome the size problem, a useful approach is to isolate individual domains from a multidomain protein, by proteolysis or by use of recombinant DNA techniques, and to determine their structures individually (Cooke et al., 1987; Bogusky et al., 1989; Nowak et al., 1993). A drawback of this approach is that the isolated domain must retain a folded conformation, which is not always trivial when dealing with protein fragments. Most proteins are only marginally stable at ambient temperature, and typically begin to unfold at 50–60 °C. A slight reduction in stability can be sufficient to result in unstructured, or only partially structured, material. Studies of mutant proteins have shown that even single amino acid substitutions are often accompanied by large decreases in unfolding temperature (Eriksson et al., 1993; Fersht and Serrano, 1993). It is,

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therefore, not surprising that subfragments of a protein should be less stable than the original, and in some instances insufficiently stable to give a useful spectrum.

Problems caused by protein instability are not confined to spectroscopy, and occur in vivo due to environmental stresses such as heat or high denaturant concentrations. Various organisms have evolved a mechanism to counter this by accumulating high concentrations of certain low-molecular-weight compounds, known as osmolytes. Osmolytes have been shown to confer stability to enzymes without affecting either their structure or biological activity (Santoro et al., 1992). The mechanism by which these substances act is not well understood, but it is generally believed to involve an entropic effect due to preferential hydration of the protein. This phenomenon opposes an increase in surface area exposed to the solvent, and therefore shifts the native $\rightleftharpoons$ unfolded equilibrium towards the native state (Arakawa and Timasheff, 1983). However, irrespective of the mechanism, it appears that the effect is applicable to any protein, and thus provides a general method of stabilizing their folded structure, with unfolding temperatures raised by as much as 23 °C (Santoro et al., 1992). Here we report how such osmolytes can be used to good effect to stabilize proteins during NMR experiments.

Some of the most efficient osmolytes reported are the glycine-based derivatives that include glycine, sarcosine and betaine. For NMR purposes it is advantageous to use deuterated material,

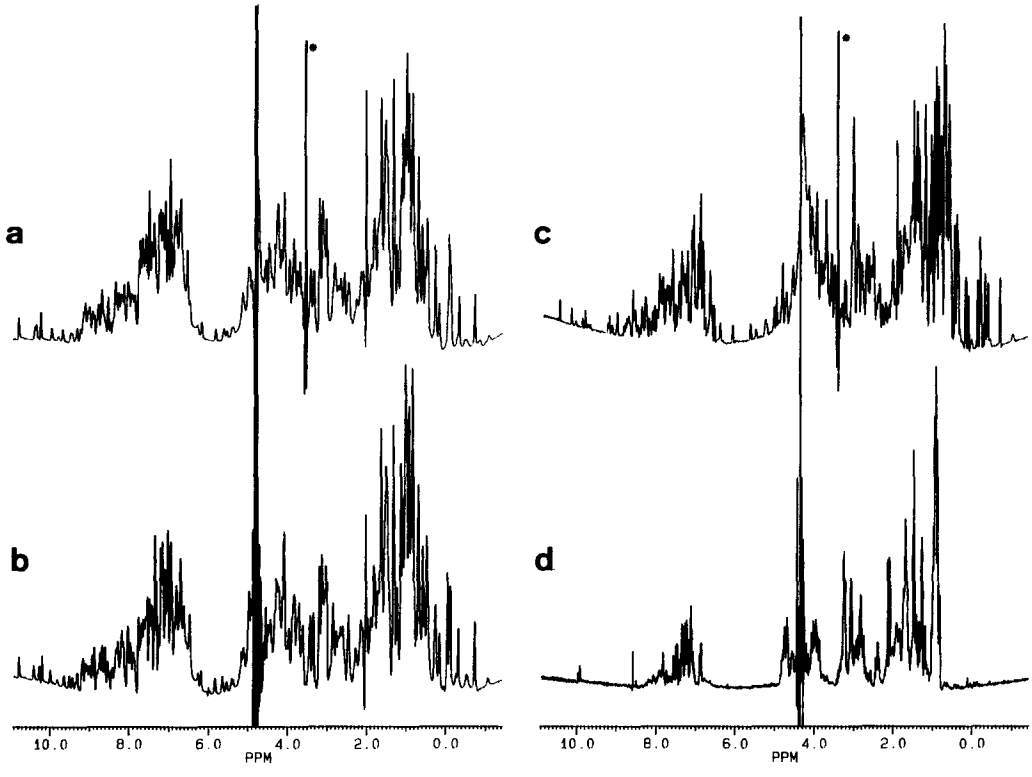

Fig. 1. 500.13 MHz ^{1}H spectra of lysozyme at pH 4.0 in (a) 9:1 H_2O:D_2O with 2M perdeuterated glycine, 298 K; (b) 9:1 H_2O:D_2O, 298 K; (c) 9:1 H_2O:D_2O with 2 M perdeuterated glycine, 348 K; (d) 9:1 H_2O:D_2O, 348 K. The 1D ^{1}H spectra were acquired over 32K data points and the FIDs were resolution-enhanced prior to Fourier transformation. The resonance due to H_2O was suppressed by presaturation. Asterisks indicate the residual $C_\alpha H$ peak of the glycine solute.

and we have selected glycine because it is readily available in deuterated form. Figures 1a and b show the ^{1}H spectra of hen egg-white lysozyme (Sigma Chemical Co.) in aqueous 2M perdeuterated glycine (glycine-d_5, 98 atom-%, Sigma Chemical Co.) and in water at 298 K. Lysozyme is a well-characterised globular protein with a fully assigned proton spectrum (Redfield and Dobson, 1988; Smith et al., 1993). It can be seen that glycine, up to 2 molar, has a negligible effect on the intrinsic quality and nature of the NMR spectra. As ^{1}H chemical shifts are a sensitive probe of protein conformation, this shows that the high glycine concentration has minimal effects on the protein conformation. This is entirely consistent with previous studies that show no influence of osmolytes on biological activity (Santoro et al., 1992). Figures 1c and d show the result of raising the temperature to 348 K. Under these conditions, lysozyme ceases to be folded in the absence of the osmolyte, which is immediately evident from the lack of dispersed resonances in Fig. 1d. However, in the presence of the osmolyte, the folded conformation of lysozyme is maintained (Fig. 1c). At this temperature it is impossible to record meaningful 2D spectra in water, but high-quality spectra are obtained in the presence of 2M glycine. This is illustrated in Fig. 2 which shows the 2D ^{1}H COSY 'finger print' region of lysozyme with glycine at 348 K. An additional benefit of recording spectra at elevated temperatures is the reduction in linewidths, evident in Fig. 1d, which markedly improves the resolution.

In summary, the potential uses of osmolytes for NMR are twofold. First, they can allow record-

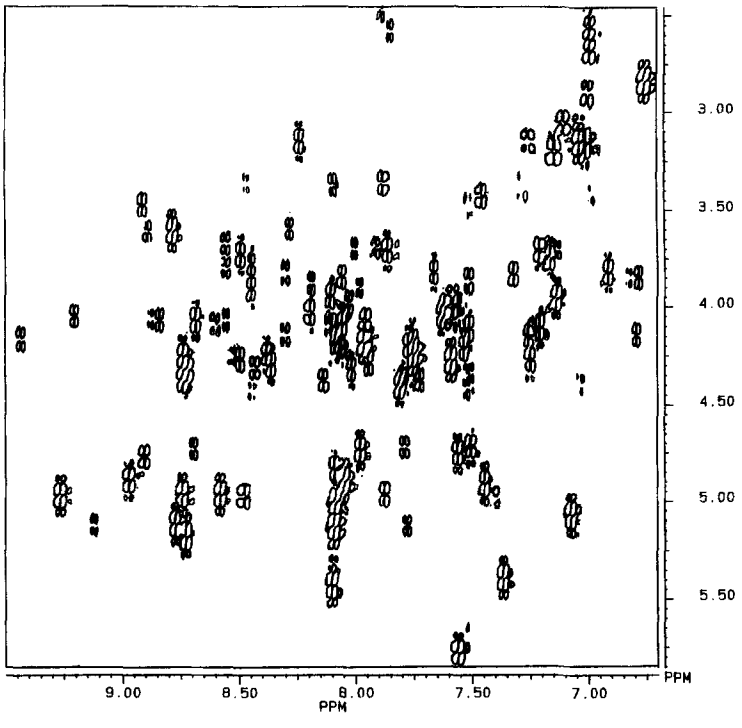

Fig. 2. 500.13 MHz 2D ^{1}H COSY spectrum of lysozyme at pH 4.0 in 9:1 H_2O:D_2O with 2M perdeuterated glycine, 348 K. The 2D ^{1}H COSY spectrum was acquired over 2K data points and 380 t_1 increments. The dataset was multiplied by a sine-bell window function in both dimensions. After zero-filling, the digital resolution was 3.6 and 1.8 Hz/pt in the F1 and F2 dimensions, respectively. The resonance due to H_2O was suppressed by presaturation.

ing of spectra of stable proteins under more stringent conditions, such as elevated temperatures, Second, and potentially more importantly, they can provide a means whereby unstable proteins are stabilized to the extent that useful NMR spectra may be recorded. We envisage that this technique will be of value to structural studies involving protein fragments or mutant proteins.

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