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Appropriateness of DSS and TSP as internal references for ^{1}H NMR studies of molten globule proteins in aqueous media

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SUMMARY

A marked dependence of the ^{1}H resonances of TSP and DSS (internal standards) on the concentration of proteins in the molten globule state has been found. This result indicates that TSP and DSS interact with these proteins. Therefore, when the chemical shift is used as an indicator of the residual structure of proteins in the molten globule state, great care must be taken in using TSP and DSS.

3-Trimethylsilylpropane sulfonic acid (DSS) and 2,2,3,3-tetradeutero-3-trimethylsilylpropionic acid (TSP) have been thought to be appropriate as internal chemical shift standards for studies on protein conformation in aqueous media. However, we have found that, in the ^{1}H NMR spectrum of bovine α-lactalbumin (BLA) at pD 2.0, the chemical shifts of DSS and TSP with respect to the HDO resonance depend considerably on the concentration of BLA. At pH 2.0, BLA is in the molten globule (MG) state, i.e., a compact conformation with the native (N)-like secondary structure, but without the side-chain packing seen in the N state (Kuwajima, 1989; Ptitsyn, 1992). The MG state has been characterized as one of partially unfolded states of proteins, which accumulates at the early stage of the folding process, and its structural information is needed to clarify the mechanism of protein folding. Recently, residual structures in unfolded states of various proteins have been investigated by NMR, in which DSS and TSP were mainly used as the internal standard of ^{1}H NMR resonances (Baum et al., 1989; Evans et al., 1991; Buck et al., 1993; Cox et al., 1993; Peng et al., 1993; Shimizu et al., 1993; Stockman et al., 1993; Van Mierlo et al., 1993; Alexandrescu et al., 1993,1994a,b; Logan et al., 1994; Wüthrich, 1994). Although the chemical shift is one of the easily and most accurately measurable indicators of the residual structure, its accuracy depends on the inert character of internal standards. Because we have doubted the validity of use of TSP and DSS as the standard in the ^{1}H NMR spectra of MG

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proteins, here we discuss their appropriateness as the internal reference for ^{1}H NMR spectra of proteins in the N, MG and fully unfolded (U) states.

The proteins used in this study are BLA, hen egg white lysozyme (Lyz), and oxidized horse heart cytochrome *c* (Cyt *c*). BLA is one of the globular proteins in which the MG state is well characterized, especially at acidic pH (Baum et al., 1989; Alexandrescu et al., 1993,1994b; Shimizu et al., 1993). Lyz has an amino acid sequence similar to that of BLA (Nitta and Sugai, 1989; Evans et al., 1991), but the equilibrium MG state has not been detected at any pH. Cyt *c* also assumes the MG conformation at acidic pH, depending on salt concentration (Goto et al., 1990). The internal references tested in this study are TSP, DSS and dioxane. These have been used as the internal reference for ^{1}H NMR spectroscopy in aqueous media, and the pH dependence of the shifts has been studied in detail (De Marco, 1977). Here, dependence of the chemical shifts of the references on protein concentration was measured in aqueous solutions of the proteins at pD 2.0 and 6.8. BLA and Cyt *c* are in the N state at pD 6.8 and in the MG state at pD 2.0. Lyz is in the N state at both pD 6.8 and 2.0.

The resonance frequency of the HDO peak in the protein solutions was independent of protein concentration when pH and salt concentration were fixed. By using the HDO resonance as a standard, the chemical shifts and line widths of the ^{1}H resonances of BLA were observed to be independent of BLA concentration at pD 6.8. Under this condition, the chemical shifts of DSS, TSP and dioxane are also independent of BLA concentration (Fig. 1A). At pD 2.0, however, the resonances of TSP and DSS are markedly dependent on the protein concentration (Fig. 1B). The dioxane resonance is independent of protein concentration, even at pD 2.0. Similar results were obtained for Cyt *c* (Figs. 1C and D). These results indicate that TSP and DSS interact with proteins in the MG state. It is well known that the MG state binds a hydrophobic dye, ANS (1-anilinonaphthalene-8-sulfonic acid) (Kuwajima, 1989; Semisotnov, 1991; Ptitsyn, 1992). The resonances of TSP and DSS, however, are dependent on Lyz concentration at both pD 2.0 and 6.8, where Lyz assumes the N state (Figs. 1E and F). Therefore, the interactions of TSP and DSS with proteins are not restricted to the MG state. It has been reported that Lyz in the N state binds ANS (Cardamone and Puri, 1992). Also, DSS has been found to be an unsuitable reference compound for ^{1}H chemical shift studies in aqueous solutions containing aromatic solute molecules (Lam et al., 1977). In typical ^{1}H NMR experiments, ~1–5 mM protein solutions have been used. At 3 mM of BLA or Cyt *c*, deviations of the chemical shifts of TSP and DSS from the values in the absence of protein are approximately −0.08–−0.16 ppm at pD 2.0. These values are small but significant, because the deviations of the chemical shift from the random coil value, which have been used as an indication of residual structures in unfolded proteins, are ~0.1–0.3 ppm (Baum et al., 1989; Evans et al., 1991; Buck et al., 1993; Cox et al., 1993; Peng et al., 1993; Shimizu et al., 1993; Stockman et al., 1993; Van Mierlo et al., 1993; Alexandrescu et al., 1993,1994a,b; Logan et al., 1994; Wüthrich, 1994). Our results suggest that DSS and TSP are not appropriate as internal ^{1}H NMR standards in studies of proteins in the MG state. Dioxane may be recommended to be used for discussion on the chemical shifts of proton resonances in partially unfolded proteins, such as BLA in the MG state.

There are two possible reasons for the dependence of the shifts of TSP and DSS on protein concentration in aqueous solutions. One is the hydrophobic interaction between the internal references and the proteins. The protein concentration dependence of the chemical shift of DSS is generally more remarkable than that of TSP in the protein solutions (Fig. 1). As DSS has one

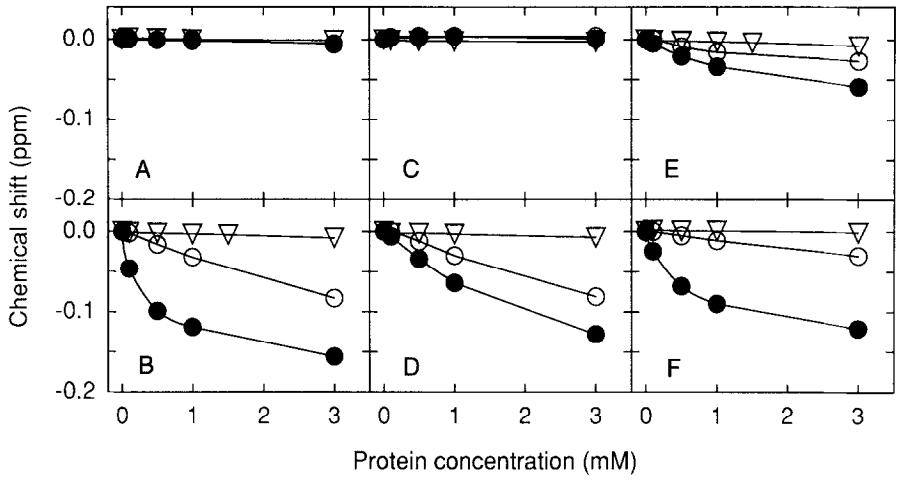

Fig. 1. Chemical shifts of TSP, DSS and dioxane resonances (1 mM) as a function of protein concentration at 35 °C in aqueous BLA solutions at pD 6.8 (A) and pD 2.0 (B), in 0.2 M NaCl aqueous Cyt *c* solutions at pD 6.8 (C) and pD 2.0 (D), and in aqueous Lyz solutions at pD 6.8 (E) and pD 2.0 (F). (○) = TSP; (●) = DSS; (▽) = dioxane. The HDO resonance is used as reference. The chemical shift shows the deviation from the position of the internal reference in the absence of protein.

more methylene group than TSP, the affinity of DSS for hydrophobic regions in the proteins may be larger than that of TSP. The other is the electrostatic force, as the dependence of the shift of DSS is suppressed by adding salt (data not shown). At pH 2.0, DSS has a negative charge and the proteins are positively charged, although TSP has no charge (De Marco, 1977).

If the hydrophobic binding to proteins in the MG state is one of the reasons for the shifts of TSP and DSS, these shifts are expected to be decreased by adding urea, because the MG state is converted to the U state by urea. We have investigated the dependence of the resonance frequencies of TSP and DSS in BLA solutions on urea concentration. In this study the HDO resonance cannot be used as a reference, because it shifts with urea concentration. Therefore, we used an impurity peak at 0.148 ppm from TSP at pD 2.0, which is independent of both protein and urea concentrations (Shimizu et al., 1993). The impurity resonance is observed in the spectra of commercially available D_2O in septum vial bottles and is probably caused by the silicon rubber cap (Merck and Isotec), but it is not observed in the spectra of D_2O from ampuls. Judged by the chemical shift, the impurity may be a silicon compound. The amount of impurity is very small. The impurity peak seems to be appropriate as a new reference for protein solutions, although the substance corresponding to the impurity peak has not been identified. Figure 2 shows the urea concentration dependence of the chemical shifts of TSP and DSS in an aqueous solution of 1 mM BLA. As expected, the resonances of TSP and DSS show a shift to lower field with up to 6 M urea; at higher urea concentrations, the chemical shifts approach the standard values. From Fig. 2 we conclude that TSP and DSS can safely be used as internal references for the chemical shifts of unfolded protein in the presence of urea.

In conclusion, dioxane is an appropriate internal reference for investigations of the chemical shifts of protons in proteins, irrespective of their conformations, in aqueous solutions, although the chemical shift of dioxane overlaps with those of C^{β} protons in protein molecules. The dioxane

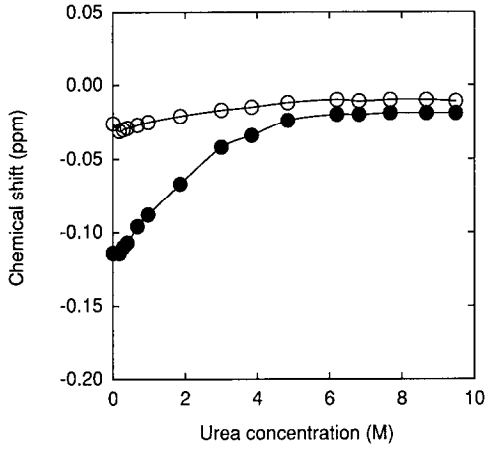

Fig. 2. Chemical shifts of TSP and DSS resonances (0.1 mM) in aqueous solutions of 1 mM BLA as a function of urea concentration (pD 2.0, 35 °C). (○) = TSP; (●) = DSS. An impurity resonance (see text) was used as reference (0.148 ppm).

resonance in the protein solution is independent of both protein concentration and pH. When using TSP or DSS as internal standards to study the MG state, especially when the chemical shift is used as an indication of structure, great care must be taken.

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