Denaturation Studies by ^{13}C Nuclear Magnetic Resonance on Modified Basic Pancreatic Trypsin Inhibitor Using the Novel *S*-[^{13}C]Methyl Methionyl Probe

Benjamin M. Harina,[1a] Douglas F. Dyckes,*[1a] M. Robert Willcott, III,*[1a] and Warren C. Jones, Jr.[1b]

Contribution from the Department of Chemistry, University of Houston—Central Campus, Houston, Texas 77004, and the Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901. *Received May 16, 1979*

Abstract: The single methionine residue (Met-52) of basic bovine pancreatic trypsin inhibitor (BPTI) has been methylated to yield the diastereomeric *S*-[^{13}C]methyl methionyl inhibitor (SM-BPTI) which retains full activity and shows conformational stability similar to unmodified BPTI. The diastereomeric *S*-[^{13}C]methyl groups can be distinguished spectroscopically by ^{13}C nuclear magnetic resonance spectroscopy. The spectrometric nonequivalence was 0.2 ppm. *S*-[^{13}C]Methyl methionine itself and selected substituted derivatives also showed a measurable but smaller chemical shift separation in the methyl signals (0.02–0.09 ppm). The observed nonequivalence in the *S*-methyl amino acid compounds was found to be dependent upon the state of ionization of the amino acid and the nature of the substituent group. Comparison of the spectral parameters of the *S*-methyl label in the protein with those of the *S*-methyl label in the amino acid model compounds has led to the conclusion that the observed nonequivalence in the methyl groups of the protein is due to effects of environmental and conformational factors. The sensitivity of the label to these factors has been demonstrated in studies of the protein performed under the different conditions of pH, temperature, and salt concentration. Comparison of the changes in the mean chemical shift with the absolute nonequivalence of the methyl signals in the protein observed under the different perturbing conditions has also provided valuable insights into the solution conformation of the protein.

Methionine residues in most proteins provide convenient sites for specific enrichment with ^{13}C isotopes. The relatively low proportion of these residues gives rise to ^{13}C nuclear magnetic resonance spectra (^{13}C NMR)[1] which appear to be uncomplicated. Specific enrichment with ^{13}C isotopes has an inherent advantage in ^{13}C NMR. The signal from protein label dominates the spectrum and circumvents the need to separate the unlabeled protein. The signals from the ^{13}C-enriched proteins reveal particular events that affect the label when the protein in solution undergoes conformational changes on denaturation or ligand binding. One labeling technique, which serves to increase the presently limited application of ^{13}C NMR to proteins, is the S-methylation of methionyl residues with $^{13}CH_3I$, a modification resulting in a positively charged sulfonium group. Recent investigators have used the ^{13}C *S*-methyl sulfonium as an intermediate in producing the specifically enriched ^{13}C methionine form of the protein. Jones et al.[2,3] reported the ^{13}C enrichment of the two methionine residues in sperm whale myoglobin by demethylation of the ^{13}C-enriched S-methylated protein. Similarly, Deber et al.[4] reported spin–lattice relaxation measurements in solution on the ^{13}C-enriched S-methylated form and the ^{13}C-enriched methionine form of the human and bovine myelin basic proteins. The methylation of the single methionine residue of glucagon has also been reported.[5]

The single methionine residue (Met-52) of the basic bovine pancreatic trypsin inhibitor (BPTI) which lies in the center of the C-terminal helix of the molecule[6] can be methylated with $^{13}CH_3I$ in 70% formic acid to yield the two diastereomeric forms of *S*-[^{13}C]methyl methionyl BPTI (SM-BPTI). The modified protein retains full activity and, as we shall demonstrate, displays conformational stability similar to BPTI under the different denaturation conditions of high pH, high temperature, or high salt concentration.

We reported in a preliminary communication that the diastereomers of SM-BPTI can be distinguished spectroscopically by ^{13}C NMR and that the chemical-shift separation of the ^{13}C methyl signals provides a useful and sensitive probe for monitoring conformational changes in the protein.[7] The spectroscopic resolution of the resonances from the diastereomers in an *S*-methyl methionyl containing protein had not been previously observed. We now report the results of further investigations which have identified factors contributing to the nonequivalence of the methyl signals in SM-BPTI.

Experimental Section

A. Preparation of the *S*-[^{13}C]Methyl Methionine and Its Derivatives. 1. *S*-[^{13}C]Methyl L-Methionine (SM-Met), *S*-[^{13}C]Methyl L-Methionine Amide (SM-Met-NH_2), *S*-[^{13}C]Methyl L-Methionine Methyl Ester (SM-Met-OCH_3). L-Methionine, 120 mg in 2.5 mL of H_2O, was reacted at room temperature in the dark with 0.08 mL of 90% enriched $^{13}CH_3I$ (Prochem, Summit, N.J.) in a tightly closed vial with Teflon-lined screw cap. The amino acid readily went into solution with stirring after 30 min. The reaction was allowed to proceed for another 1 h; then excess $^{13}CH_3I$ was allowed to evaporate naturally from the reaction vessel. A clear solution of SM-Met was obtained and was used directly for the ^{13}C NMR studies. SM-Met-NH_2 and SM-Met-OCH_3 were prepared in the same manner from L-methionine amide hydrochloride and L-methionine methyl ester hydrochloride (Sigma, St. Louis, Mo.), respectively.

2. *N*-α-*t*-Boc-*S*-[^{13}C]methyl Methionine (*t*-Boc-SM-Met). *N*-α-*t*-Boc-L-methionine (Bachem, Marina del Rey, Calif.), 150 mg in 3 mL of 0.2 M Na_2HPO_4 at pH 9, was reacted with 0.1 mL of 90% $^{13}CH_3I$ using the same procedure as 1. The solution of *t*-Boc-SM-Met was used directly for ^{13}C NMR studies.

3. *N*-α-Acetyl-*S*-[^{13}C]methyl L-Methionine (Ac-SM-Met). *N*-α-Acetyl L-methionine (200 mg) was dissolved in 3 mL of H_2O by warming and was reacted at room temperature with 0.1 mL of 90% $^{13}CH_3I$ using the same procedure as 1. The solution of Ac-SM-Met was used directly for ^{13}C NMR studies. Acetyl methionine was prepared by a method adapted from Moore and Dalrymple.[8]

4. *N*-α-Z-*S*-[^{13}C]methyl L-Methionine (Z-SM-Met) and *N*-α-Z-*S*-[^{13}C]methyl-L-methionyl-L-glycyl Ethyl Ester (Z-SM-Met-Gly-OEt). Z-Methionine, 90 mg in 2 mL of H_2O, was reacted with 0.04 mL of $^{13}CH_3I$ for 3 h using the same procedure as 1. The solution of Z-SM-Met was used directly for ^{13}C NMR studies. Z-Methionine was prepared by the method of Hofmann et al.[9] The Z dipeptide was prepared in the same manner from *N*-α-Z-L-methionyl glycyl ethyl ester (Sigma, St. Louis, Mo.).

B. Preparation of [*S*-[^{13}C]Methyl Methionine-52]-BPTI (SM-BPTI). BPTI (Trasylol, Bayer AG, Wuppertal) was used without further purification. A sample of 145 mg of BPTI dissolved in 2 mL of H_2O was added to 8 mL of 88% formic acid and allowed to react with 0.1 mL of $^{13}CH_3I$ overnight at room temperature in the dark. Excess acid was neutralized with 2 N NaOH and the solution was

0002-7863/80/1502-1120$01.00/0

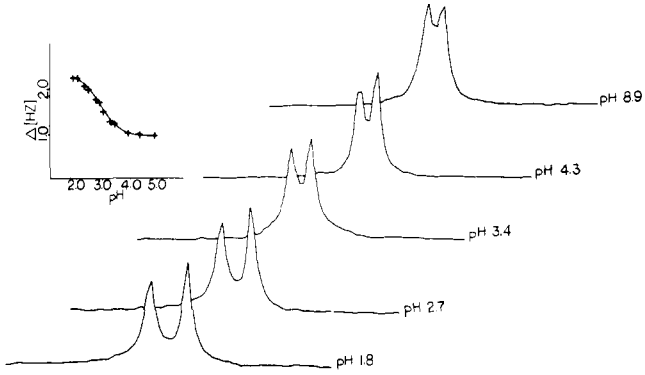

Figure 1. Successively offset 20-Hz segments of the proton-decoupled 25.16-MHz FT ^{13}C NMR spectra of 0.2 M *N*-Z-*S*-[^{13}C]methyl methionine at indicated pH values (uncorrected). There are more points in the graph than shown in the stacked plot of the ^{13}C NMR spectra. The conditions of acquisition are reported in the Experimental Section.

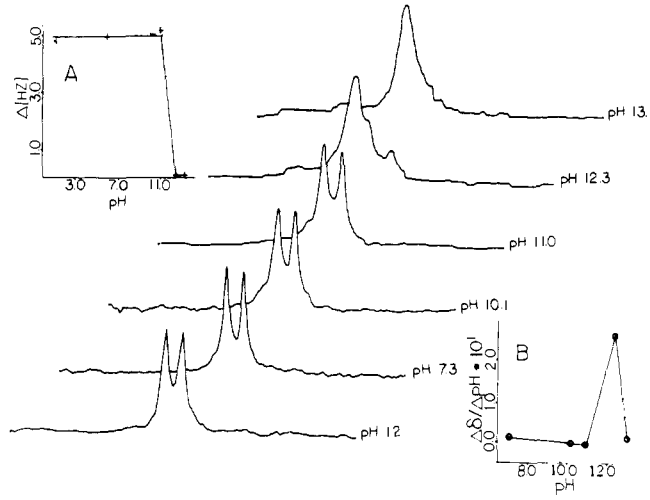

Figure 2. Successively offset 100-Hz segments of the proton-decoupled 25.16-MHz FT ^{13}C NMR spectra of 1.8 mM [*S*-[^{13}C]methyl methionine-52]-BPTI at indicated pH values (uncorrected). (A) The plot of the chemical-shift separation of the methyl signals against pH. (B) The plot of the change (ppm) in the mean chemical shift of the methyl signals per unit change in pH against pH. No other signals were observed in the full (5000 Hz) spectra except for dioxane, added as an internal standard.

dialyzed three times in 4 L of distilled H_2O in tubing with 3500 molecular weight cutoff (Arthur Thomas Co., Philadelphia, Pa.). The salt-free solution was freeze dried, resulting in a yield of 140 mg of SM-BPTI. Ion exchange chromatography in carboxymethylcellulose gave two peaks corresponding to 80% SM-BPTI and 20% unlabeled BPTI. The amino acid analysis of the purified SM protein (hydrolyzed for 16 h in vacuo at 110 °C in 4 N methanesulfonic acid) gave approximately one residue of SM-Met, which appeared after the lysine peak, and trace amounts of methionine. Measurements of trypsin-inhibitory potency for all samples assayed were carried out by the method of Kassell[12] using N^{α}-benzoylarginine *p*-nitroanilide as the chromogenic trypsin substrate.

C. ^{13}C NMR. All ^{13}C NMR studies were made in 12-mm tubes on a Varian XL-100/(25.16 MHz ^{13}C) NMR spectrometer with a Nicolet Fourier transform accessory and a variable-temperature probe. Except for samples in guanidine hydrochloride (Gu-HCl), solutions were made in 1.5 mL of H_2O and 0.5 mL of D_2O for deuterium locking. An external coaxial 5-mm tube with D_2O was used for deuterium locking for samples in Gu-HCl in H_2O. *p*-Dioxane was employed as the internal reference for all samples. The chemical shifts were corrected to the δ scale (trimethylsilylpropionic acid, TSP = 0) using a chemical shift of 68.60 ppm for dioxane in aqueous solution. Except for samples in Gu-HCl, all sample tubes were fitted with a vortex plug.

The pH titrations of the SM-Met and its derivatives were done serially by starting at low pH. The samples for the pH titration of the SM-BPTI were made by dissolving 35 mg of the protein at each specified pH and allowing the solution to stand for 2 days before taking the ^{13}C NMR spectrum. The pH was adjusted with 1 N HCl or 2 N NaOH using values uncorrected for deuterium isotope effects and sodium ion interference at high pH. The pH values measured before and after ^{13}C NMR spectra were recorded did not differ significantly.

Variable-temperature studies were performed on a single sample of 50 mg of SM-BPTI at pH 6.8 in increments of 5 °C in temperature starting at probe temperature. The sample was allowed to equilibrate at the specified temperature for 30 min before the ^{13}C NMR spectrum was taken. At each temperature, the spectrum was taken twice to ascertain that equilibrium was established. Studies in Gu-HCl (0–4 M) were made on a single sample of 50 mg of SM-BPTI starting in aqueous solution. Calculated amounts of 8 M Gu-HCl solution (pH 7.5) were added to increase the salt concentration. Another 50-mg sample of protein was prepared at 5 M salt to avoid dilution problems at high salt concentrations.

The NMR acquisition parameters were routinely set to obtain 0.12-Hz digital resolution for the experiments using the SM amino acids and 1.0-Hz digital resolution for the SM protein. The coalesced methyl signals from the SM protein were remeasured at 0.12-Hz digital resolution to check if the signals could be further resolved. The pulse width for a 90° pulse in the instrument is 19 μs; 12-μs pulses and 0.1-s delay were used in the experiments. Usually, 16 scans (2.2 min, interpulse delay = 8.4 s) were taken for each 20 mg of the ^{13}C SM amino acids and 15 000 pulses (4.6 h, interpulse delay = 1.1 s) for 50 mg of the ^{13}C-enriched protein.

Results

An illustrative example of the pH dependence of the magnitude of the chemical-shift separation of the *S*-methyl groups in amino acid model compounds is the titration of N-Z-SM-Met shown in Figure 1. The maximum chemical-shift separation is 0.09 ppm at pH values well below the apparent pK_a (~3.0) of the carboxylate group. This decreases to 0.04 ppm above pH 4.[13] Two other N-substituted *S*-methyl amino acids, N-α-*t*-Boc-SM-Met and *N*-α-acetyl-SM-Met, also exhibited signals which titrated with the carboxylate function. These showed maximum chemical-shift separations of 0.08 and 0.03 ppm, respectively, at low pH values but complete coalescence of the methyl signals in each case at values significantly above the carboxylate group pK_a's. The methyl groups of unsubstituted SM-Met showed maximum chemical-shift separation (0.04 ppm) at pH values between the pK_a values of the carboxylate and amine functions. Below pH 2 or above pH 8 the signals were unresolved. The amide and methyl ester of SM-Met were also studied, but no resolution of the methyl signals could be observed at 25.16 MHz[14] in the pH range 2–10. The Z-substituted dipeptide, Z-*S*-[^{13}C]methyl methionyl glycine ethyl ester, showed a resolution of 0.05 ppm in the methyl signals, invariant in the pH range 2–9. All pH titrations of SM-Met and its derivatives were fully reversible, and the mean chemical shifts of the methyl signals (26.8–26.9 ppm) remained invariant in the titration range.

The pH denaturation profile of SM-BPTI is shown in Figure 2. The nonequivalence of the protein-borne *S*-methyl signals was 0.20 ppm, three to eight times larger than in SM-Met and its substituted derivatives. In contrast with the *S*-methyl amino acids, but like the fully protected dipeptide, the diastereotopic separation in BPTI remained invariant in the pH range 1–11. Complete coalescence was observed only above pH 13. The mean chemical shift (26.52 ppm) remained invariant from pH 6 to 11 and moved downfield by 0.3 ppm with full coalescence at pH 13. The protein at pH 13 precipitated on dialysis with distilled water. The soluble fraction had an activity of 13% with respect to native BPTI and the ^{13}C NMR at pH 7 gave only a single broad resonance at 26.72 ppm—0.2 ppm downfield from the active protein at the same pH. In a control experiment BPTI maintained at pH 12.8 for 2 days also showed precipitation on dialysis. The soluble fraction was 52% active.

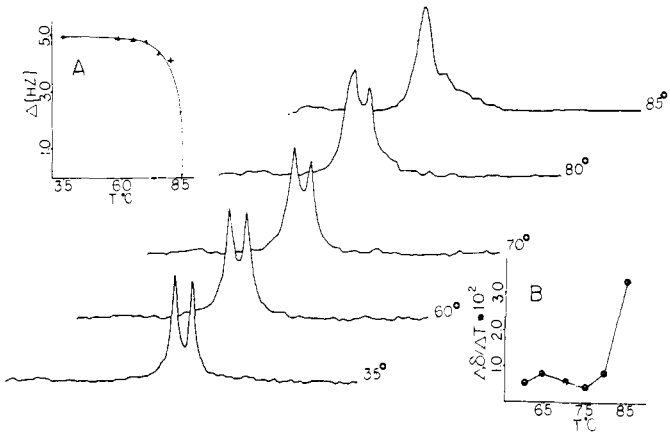

Figure 3. Successively offset 100-Hz segments of the proton-decoupled 25.16-MHz FT ^{13}C NMR spectra of 3.8 mM [*S*-[^{13}C]methyl methionine-52]-BPTI at indicated temperatures. (A) The plot of the chemical-shift separation of the methyl signals against temperature. (B) The plot of the change (ppm) in the mean chemical shift of the methyl signals per unit change in temperature against temperature.

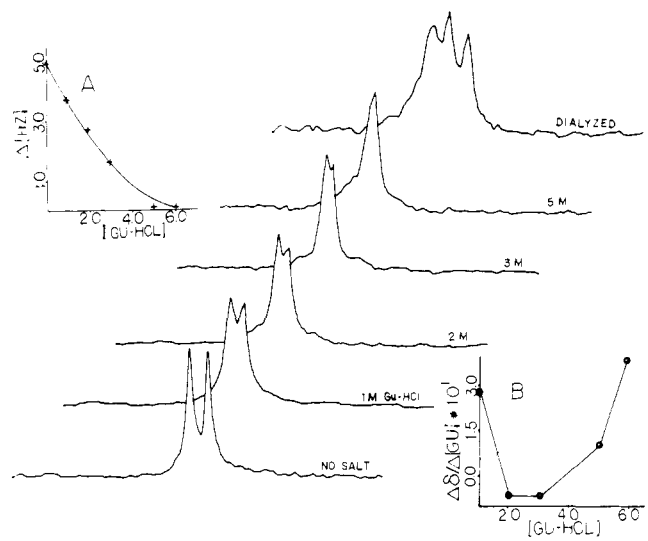

Figure 4. Successively offset 100-Hz segments of the proton-decoupled 25.16-MHz FT ^{13}C NMR spectra of 2.3 mM [*S*-[^{13}C]methyl methionine-52]-BPTI at indicated guanidine hydrochloride concentrations. Dialyzed is a protein sample that was restored to low salt solution by dialysis after 1 month in 6 M salt. (A) The plot of the chemical-shift separation of the methyl signals against guanidine hydrochloride concentration. (B) The plot of the change (ppm) in the mean chemical shift of the methyl signals per unit change in salt concentration against salt concentration.

The temperature denaturation of SM-BPTI (Figure 3) similarly showed a sharp melting profile. The chemical-shift separation of the methyl signals was still 4.1 Hz at 80 °C, compared to 4.9 Hz at 35 °C. At 85 °C, the signals coalesced. The mean chemical shift moved gradually downfield by 0.37 ppm from 35 to 80 °C and by 0.2 ppm from 80 to 85 °C. When this sample was maintained at room temperature for several weeks, no spectral separation of peaks could be observed. Instead, a single peak was observed at 26.80 ppm—0.3 ppm downfield from the mean resonance of the active protein. The heat-treated protein was 18% active, while a control solution of BPTI heated at 85 °C for 7 h and allowed to cool was 26% active.

The behavior of SM-BPTI at pH 7.5 in varying concentrations of guanidine hydrochloride is shown in Figure 4. The chemical-shift separation decreased gradually on going from the aqueous solution to higher salt concentration. It collapsed in 5 M salt accompanied by a 0.4-ppm downfield shift. The protein was brought further to 6 M salt and, after standing for 4 weeks, it was dialyzed repeatedly against distilled water. The activity was 57% and the ^{13}C NMR (Figure 4, uppermost trace) gave a downfield peak at 26.83 ppm corresponding to denatured SM-BPTI and a pair of peaks centered at 26.54 ppm corresponding to those of undenatured SM-BPTI. Control BPTI subjected to similar conditions was 64% active. In another preparation where SM-BPTI was allowed to stand in 6 M salt for only 1 week, the protein was 86% active after removal of the salt. SM-BPTI that stood in 5 M salt for 4 days showed complete resolution of the methyl signals after removal of the salt.

Discussion

Sulfonium ions do not undergo the rapid inversion characteristic of ammonium ions, despite the presence of an unshared pair of electrons. Thus the two methyl groups of *S*-methyl methionine are diastereotopic. In this work, where ^{13}C-methylation was used, the two methyl groups are isotopically distinguishable and therefore diastereomeric *S*-methyl methionyl derivatives are formed. The separate signals observed in the ^{13}C NMR spectra represent the single ^{13}C methyl group of each diastereomer.

Studies on derivatives of *S*-methyl methionine, used as models for SM-BPTI, show that the magnetic nonequivalence of the diastereomeric methyl groups is highly sensitive to the charge state of the α-amine and α-carboxyl functions, and somewhat less sensitive to the nature of amine blocking groups. The exception to this was seen in the case of *Z*-blocked SM-Met. Here, the aromatic substituent apparently causes a nonequivalence of approximately 0.04 ppm independent of the protonation state of the amino acid.

The ^{13}C methyl groups of SM-BPTI also exhibit a magnetic nonequivalence which is independent of pH throughout the range associated with titration of the carboxyl and amine functions of proteins. This nonequivalence is three to eight times larger than that observed in any of the SM-Met models and probably reflects the fact that in the native conformation of BPTI[6] the methionyl residue is held close to a hydrophobic region containing several aromatic residues (Tyr-21, Tyr-23, Phe-4, Phe-22, and Phe-45). Under conditions of pH and temperature shown by others[15,16] using independent NMR parameters, to denature BPTI, the large nonequivalence of the SM-BPTI methyl groups disappears. Denaturation almost certainly removes the methionine from close contact with the aromatic residues, and the enhancement of nonequivalence is lost. Denaturation by pH or temperature is also associated with a sharp change in the chemical shift of the SM-BPTI methyl signals (see insets B to Figures 2 and 3). Nevertheless the signals of the methyl groups in the denatured proteins are still in the range associated with the methyl sulfonium group. It is therefore unlikely that the observed spectral changes represent a chemical modification of the reporter group. Under both sets of conditions the denaturation reaction is irreversible, as judged by the failure of the molecule to regain either its original ^{13}C NMR spectrum or its inhibitory potency. BPTI is also irreversibly denatured under these conditions.

The pH- and temperature-induced changes are different from the structural changes that occur when the SM protein is carboxymethylated at all cysteine residues by treatment with iodoacetic acid in 6 M guanidine hydrochloride and excess dithioerythritol. The carboxymethylated protein, which is presumably in a random coil structure, exhibited a well-resolved chemical-shift difference of 0.08 ppm in the *S*-methyl methionyl residue at pH 1 in aqueous solution.[15] This value is comparable to the values obtained for the amino acid model compounds.

The behavior of SM-BPTI in varying concentrations of guanidine hydrochloride is again different (Figure 4). There

is a monotonic decrease in the separation of the *S*-methyl signals when the salt concentration is increased as opposed to the abrupt collapse of the signals observed at high temperature or high pH. Although it is possible that the reduction in the nonequivalence of the methyl signals is due to the interaction of the label with the salt, consideration of the ratio of the salt to protein concentration which is already very large at 1 M salt (400:1) leads us to conclude that these interactions would be expected to have reached saturation. The further reduction of nonequivalence at higher salt concentration and total coalescence at 5 M salt are probably due to local conformational changes which are reversible, since the native spectrum is restored when the salt is removed. Accordingly, the S-methylated protein is not fully inactivated by the salt, in contrast to the observed irreversible inactivation of the protein by high pH or high temperature. Protein that had been kept for 1 week in 6 M salt still maintained 86% activity after removal of the salt and one sample kept in 6 M salt for 1 month could be restored to 56% of its original activity. The latter preparation had both active and inactive protein which can be distinguished by ^{13}C NMR. The unresolved *S*-methyl signals of the inactive protein lie 0.3 ppm downfield from the mean chemical shift of the well-resolved signals from the active protein (Figure 4). In another preparation of the S-methylated protein that stood in 5 M salt for 4 days, the ^{13}C methyl signals were unresolved but removal of the salt by dialysis resulted in the complete resolution of the peaks and restoration of activity. These results are fully consistent with the reported stability of unmodified BPTI[16-19] in high salt concentration where denaturation is not observed unless a reducing agent is added [20] or the protein in 6 M salt is further heated to 75 °C.[21]

The mean chemical shifts of the C-13 methyl signals are also useful indicators of protein structure. To isolate the effects of perturbing conditions such as pH, temperature, or salt on chemical shift from the effects due to changes in protein structure, the change in mean chemical shift per unit change in the perturbing factor has been plotted against the magnitude of the perturbing factor. The chemical-shift contributions due to increments in the perturbing factor are small compared to the chemical-shift change induced by the protein undergoing a conformational change. The change in protein structure is reflected as the steep rise or spike in the figures (Figures 2–4, inset B). In the pH titration of SM-BPTI, the spike shows at pH 12 simultaneous with the collapse of the methyl signals. In the temperature study of SM-BPTI, the appearance of the spike at 85 °C is also coincident with the collapse of the methyl signals. Similarly, the loss of nonequivalence coincides with the largest chemical-shift change per unit salt added. The large chemical-shift change at 1 M salt, however, is assumed to be due to the sudden increase in ionic strength from aqueous solution. Changes in the mean chemical shift and in the chemical-shift separation of the methyl signals provide similar information regarding the conformational state of the protein. There is added information, however, that the chemical-shift separation provides which is not reflected in the changes in mean chemical shift. In the temperature study of SM-BPTI, the chemical-shift separation of 4.8 Hz was invariant between 35 and 70 °C. A significant reduction in the nonequivalence is noticeable in the measurements at 75 and 80 °C before full collapse is observed at 85 °C. There is no indication, however, of any significant deviation from the normal change in chemical shift per degree at or below 80 °C. Between 80 and 85 °C the change in mean chemical shift also changes markedly. These observations suggest two possible events occurring between 70 and 80 °C. One possibility is that the protein undergoes subtle structural transitions which can significantly affect the nonequivalence of the *S*-methyl groups but not their chemical shifts. The other possibility is that significant quantities of denatured protein are present in the sample. This latter possibility is supported by activity measurements on samples of the protein maintained at the experimental temperatures. The extent of inactivation under these conditions was as much as 30%. The ^{13}C NMR spectrum for the sample at 80 °C (Figure 3) suggests that a mixture of the native and denatured protein exists, indicated by the apparent broadening of the low-field peak in the doublet. Such differences would not be readily detected by a probe (e.g., [^{13}C]methionine) for which only the chemical-shift data can be monitored.

The advantage in the use of the chemical-shift separation of the *S*-methyl signals over the use of the mean chemical shift is further demonstrated in the study of SM-BPTI at varying Gu-HCl concentrations. Since the *S*-methyl groups are expected to interact with the external environment to the same extent, the variation in their chemical-shift separation reflects purely internal perturbations in the protein. Thus the observed monotonic decrease in the chemical-shift separation as a function of the salt concentration (Figure 4, inset A) would indicate the gradual loosening of protein structure. This is not clearly apparent from the chemical-shift data, even after it has been transformed in an effort to eliminate chemical-shift contributions from solvents effects (Figure 4, inset B).

Not only is the denaturation process more clearly indicated in the plot of the chemical-shift separation of the *S*-methyl signals vs. salt concentration, but the result is also more certain. The two methyl signals serve to reference one another, and only the relative change is interpreted. The dioxane reference used for measurements of absolute chemical shift may not experience solvent effects in parallel with the *S*-methyl groups in the protein. Thus observed trends based on chemical-shift data would have to be verified using some other parameters.

In conclusion, this study brings to the field of ^{13}C NMR a new probe, the *S*-[^{13}C]methyl methionyl label, applicable to other methionine-containing proteins and a useful and sensitive reporter of conformation changes in the molecule. The methylation of a methionine residue introduces into a protein an additional positive charge and a slight increase in bulk. These factors could effect substantial differences in the molecular structure of the modified and the unmodified proteins. However, the results of our studies on BPTI indicate that the conformation and stability of this protein have not been significantly perturbed by the SM modification. All indications are that the labeled BPTI remains as active and as stable to denaturing conditions as the modified protein. Previous modifications of the methionyl residue[11,22] have also shown no detectable effects on the trypsin inhibiting properties of BPTI and only slight effects in its capacity to renature.[23] Refolding studies of reduced SM-BPTI show that its rate of renaturation is comparable with that of BPTI.[24]

The use of the *S*-methyl probe has also recently been applied to human and bovine myelin basic proteins.[4] The nonequivalence in the *S*-methyl groups was not observed, however, at 2-Hz digital resolution but the interpreted data based on T_1 measurements suggested loose overall structure about the vicinity of the methionyl residues. There are, however, a number of other methionine-containing proteins whose tertiary structures are known that would appear to be equally suitable for these studies. For those proteins which exhibit a measurable nonequivalence in their labels upon S-methylation, spectroscopic findings can be correlated with the conformational state of the protein and, thereby, provide a better understanding of their solution conformations.

Acknowledgment. This work was supported in part by U.S. Public Health Service Grant GM-22927 and Robert A. Welch Foundation Grants E-183 and E-645. The Varian XL-100/Nicolet TT-100 NMR spectrometer was purchased through NSF Grant MPS 75-06162. We wish to thank Bayer A. G., Wuppertal, West Germany, for their generous gift of BPTI

(Trasylol) and Shell Development Co., Houston, Texas, for the use of their 90-MHz ^{13}C NMR facilities. We especially wish to thank Ms. Ruth Inners for obtaining initial spectra at 25 and 90 MHz and Dr. Dale Holecek of Shell Development for his assistance in obtaining the 90-MHz spectra.

References and Notes

(1) Abbreviations used: ^{13}C NMR, ^{13}C nuclear magnetic resonance; BPTI, basic pancreatic trypsin inhibitor (bovine); SM-BPTI, [*S*-[^{13}C]methyl methionine-52]-BPTI; ppm, parts per million; Z, benzyloxycarbonyl.
(2) W. C. Jones, Jr., T. M. Rothgeb, and F. R. N. Gurd, *J. Am. Chem. Soc.*, **97**, 3875 (1975).
(3) W. C. Jones, Jr., T. M. Rothgeb, and F. R. N. Gurd, *J. Biol. Chem.*, **251**, 7452 (1976).
(4) C. M. Deber, M. A. Moscarello, and D. P. Wood, *Biochemistry*, **17**, 898 (1978).
(5) T. M. Rothgeb, B. N. Jones, D. F. Hayes and R. S. Gurd, *Biochemistry*, **16**, 5813 (1977).
(6) R. Huber, D. Kukla, A. Ruhlmann, O. Epp, and H. Formanek, *Naturwissenschaften*, **57**, 389 (1970).
(7) B. M. Harina, D. F. Dyckes, M. R. Willcott, III, and W. C. Jones, Jr., *J. Am. Chem. Soc.*, **100**, 4897 (1978).
(8) J. A. Moore and D. L. Dalrymple in "Experimental Methods in Organic Chemistry", W. B. Saunders, Philadelphia, 1976, p 255.
(9) K. Hofmann, A. Johl, A. E. Furlenmeier, and H. Kappeler, *J. Am. Chem. Soc.*, **79**, 1636 (1957).
(10) D. F. Dyckes, T. E. Creighton, and R. C. Sheppard, *Nature (London)*, **247**, 202 (1974).
(11) D. F. Dyckes, T. E. Creighton, and R. C. Sheppard, *Int. J. Pept Protein Res.*, **11**, 258 (1978).
(12) B. Kassell, *Methods Enzymol.*, **19**, 844 (1970).
(13) A 0.06-ppm separation in the C-13 methyl signals of Z-SM-Met at 25 MHz has been observed recently.[4] The pH was not reported.
(14) The methyl ester of [^{13}C]-SM-methionine at pH 10 exhibited a 0.9-Hz separation in the *S*-methyl signals at 90 MHz. Under these conditions the corresponding signal for [^{13}C]-SM-methionine amide showed an unresolved shoulder on the upfield side.
(15) The carboxymethylated SM protein was observed at low pH for reasons of solubility. The heat-denatured SM protein remains soluble at physiological pH.
(16) M. P. Sherman and B. Kassell, *Biochemistry*, **7**, 3634 (1968).
(17) E. Sach, M. Thely, and J. Choay, *C. R. Acad. Sci.*, **260**, 3491 (1965).
(18) N. M. Green and E. Work, *Biochem. J.*, **54**, 257 (1953).
(19) M. Kunitz and J. H. Northrop, *J. Gen. Physiol.*, **19**, 991 (1936).
(20) S. Karplus, G. H. Snyder, and B. D. Sykes, *Biochemistry*, **12**, 1323 (1973).
(21) A. Masson and K. Wüthrich, *FEBS Lett.*, **31**, 114 (1973).
(22) B. Kassell, *Biochemistry*, **3**, 152 (1964).
(23) T. E. Creighton, D. F. Dyckes, and R. C. Sheppard, *J. Mol. Biol.*, **119**, 507 (1978).
(24) D. F. Dyckes and T. E. Creighton, manuscript in preparation.

^{1}H, ^{23}Na, and ^{31}P NMR Studies of the Self-Assembly of the 5′-Guanosine Monophosphate Dianion in Neutral Aqueous Solution in the Presence of Sodium Cations

Marie Borzo, Christian Detellier, Pierre Laszlo,* and Agnès Paris

Contribution from the Institut de Chimie Organique et de Biochimie (B6), Université de Liège, Sart-Tilman par 4000 Liège, Belgium. *Received April 12, 1979*

Abstract: ^{1}H chemical shifts show at concentrations below 0.2 M a normal stacking process, with an apparent mean equilibrium constant of 3.8 M^{-1} at 299 K. Above a concentration of approximately 0.3 M at this temperature, an altogether different mode of self-ordering sets in. The multiplicity of H-8 resonances in the ^{1}H NMR spectra and that of phosphate resonances in the ^{31}P NMR spectra are consistent with the coexistence of two kinds of ordered structures, likely to be octamers and hexadecamers, respectively. ^{23}Na chemical shifts and line widths are also concentration dependent: below a critical concentration, they increase normally with the fraction of ion-paired sodium, just as in model systems (5′-AMP, 5′-ATP, 2′-GMP, D-ribose 5-phosphate), with binding constants of about 2 M^{-1}, at 300 K. Above a critical concentration, the ^{23}Na resonance undergoes a pronounced upfield shift, together with a considerable line broadening: these are diagnostic of a sharp disorder–order transition, which can be studied either by changing the concentration at constant temperature, or by varying the temperature at constant concentration. This disorder–order transition is highly cooperative, with a Hill coefficient of 6.1 ± 0.7. From the critical concentrations, determined either from the ^{23}Na chemical shifts or from line widths reduced to unit viscosity, a phenomenological phase-separation model yields $\Delta H° = -17 \pm 2$ kcal·mol^{-1} and $\Delta S° = -51 \pm 6$ cal·mol^{-1}·K^{-1}. The self-assembly is enthalpy driven, rather than determined predominantly by hydrophobic forces, like the normal stacking interactions. The binding of Na^{+} contributes directly to the buildup of octamers and hexadecamers from hydrogen-bonded tetramers whose central cavity includes a sodium cation.

Introduction

Alone among the nucleotides, guanosine monophosphate (GMP) arranges itself into highly ordered aggregates, in aqueous solutions.[1] The 5′ isomer (5′-GMP) not only forms gels at acidic pHs,[1-5] but also self-orders at neutral and slightly basic pH. This phenomenon was discovered in 1972 by Miles and Frazier,[6] using infrared spectroscopy. Further X-ray[7] and IR studies provided nearly conclusive evidence for a planar tetrameric arrangement of GMP molecules held together by eight hydrogen bonds. ^{1}H NMR studies reported in 1975 the high rigidity of 5′-GMP aggregates at neutral pH: energy barriers of more than 15 kcal·mol^{-1} prevent fast exchange of monomer nucleotides between sites.[8] Two groups then independently discovered by NMR that the self-assembly process depends critically on the cation present: Pinnavaia et al.[9] using ^{1}H NMR and Laszlo et al.[10-13] using ^{23}Na and ^{39}K NMR showed a potassium-selective interaction of alkali metal cations with the central cavity delimited in the tetramers by the four O_6 oxygens.

5′-GMP aggregation is related to the exceptionally high rigidity displayed by poly(riboguanylic acid) [poly(G)] in neutral aqueous solution,[14-17] due to multistrand formation,[18] with a four-stranded form characterized by X-ray in the solid.[19-20]

The self-ordering of 5′-GMP could have had prebiotic significance: nucleotide formation has been documented under abiotic conditions,[21-23] and 5′-GMP self-assembles at the slightly basic pHs (7.8–8.4) characteristic of the present and plausibly also of the primeval ocean.[24-26] We have indeed already shown[27] that 5′-GMP aggregates discriminate between

0002-7863/80/1502-1124$01.00/0