Abstract  The use of diffusion coefficients measured with pulsed-field gradient NMR spectroscopy for the determination of the relative population of conformers in solutions of the human Growth Hormone peptide fragment, hGH(9–19), has been studied in aqueous and in trifluoroethanol (TFE)/water solutions. The peptide is a good model compound for this study because it adopts a predominantly random coil conformation in aqueous solution and is helical in TFE. The results of the diffusion measurements suggest that the peptide exhibits predominantly random coil structures in aqueous solution and adopts a more helical conformation in solutions containing increasing mole fractions of TFE, consistent with the qualitative findings of the standard CD and NMR experiments to probe peptide conformation. These results indicate that diffusion coefficients measured with NMR can provide additional information about temperature- and solvent-induced changes in the extent of the helical conformation for hGH(9–19) in aqueous solution and in solutions containing various mole fraction of TFE, respectively.

Introduction

The conformational analysis of peptides based on the results of NMR experiments is often complicated by the presence of rapidly interconverting random coil and ordered structures, which are reflected in the averaging of the NMR parameters [1–3]. Therefore, it is often necessary to determine not only which conformers are present but to characterize them according to their population distributions [4]. However, the determination of the relative populations of different peptide conformers under these conditions is difficult because simple analytical parameters to describe the population distribution are not readily available.

Experimental

The peptide hGH (9–19) was obtained as the lyophilized powder from either the Kansas State University Biotechnology Facilities.
Microchemical Core Laboratory or from the University of Kansas Biochemical Research Services Laboratory and used without further purification. Deuterated $d_1$-($99.5\%$ D) and $d_2$-($99\%$ D or $99.5\%$ D) 2,2,2-trifluoroethanol were purchased from Isotec Inc., Miamisburg, OH, USA and Cambridge Isotope Laboratories, Andover, MA, USA, respectively. Protonated 2,2,2-trifluoroethanol was purchased from Sigma Chemical Co., St. Louis, MO, USA. Deuterium oxide ($D_2O$), $99.9\%$ D, was purchased from either Sigma Chemical Co. or Cambridge Isotope Laboratories.

Peptide solutions were prepared by dissolving in $90\%$ H$_2$O/10\% D$_2$O or in the appropriate deuterated TFE/D$_2$O mixtures. The peptide concentration in solutions containing the various mole fractions of $d_1$-TFE and D$_2O$ ranged between 1.0 and 5.0 mM. The peptide concentration in solutions of $90\%$ H$_2$O/10\% D$_2$O generally ranged between 10.2 and 11.1 mM for these measurements. No evidence of aggregation of the peptide was detected in any of the solvent systems studied. The possibility of aggregation of the peptide was assessed by comparison of diffusion coefficients measured for these concentrated solutions with those obtained for more dilute solutions. Final peptide concentrations for CD ($83.2\mu M$) were obtained by dilution of a 2.08 mM stock solution of the peptide. The concentration of the peptide in the stock solution was determined by NMR spectroscopy as described previously [13].

CD spectra were measured using a 1.0 mm quartz jacketed cell with a 1.0 mm quartz jacketed cell equipped with a xenon lamp. The temperature was maintained within ±0.1 °C using a Brinkmann RC6 or NESLAB RTE-111 temperature regulator. CD spectra were acquired by sampling every 1 nm over the spectral range 190–250 nm at a bandwidth of 1.00 or 1.50 nm. A time average of 2.00 s for each data point was used, and five repetitions were coadded for each spectrum.

Most NMR spectra were obtained using a Bruker AM 500 MHz spectrometer and a 5 mm inverse probe equipped with a shielded z-gradient coil. One-dimensional experiments were measured using a spectral width of 6024 Hz and 16,384 data points. All NMR spectra were processed with either Felix 2.30 or 95.0. One-dimensional free induction decays (FIDs) were apodized by multiplication with a matched exponential decay corresponding to 1 or 2 Hz line broadening in the transformed spectra. The spectral baseline was corrected by fitting the baseline points to a fifth-order polynomial. 3-(Trimethylsilyl)propionic acid, 2,2,3,3-$d_4$ sodium salt (TSP) was used as an internal chemical shift reference. Solvent suppression was achieved by selective saturation of the water resonance.

Amide proton temperature coefficients for the peptide in aqueous solution were determined from the one-dimensional spectra measured over the temperature range 283 to 308 K ± 0.1 K in 90\% H$_2$O/10\% D$_2$O. Because of extensive spectral overlap in $d_1$-TFE solutions of the peptide, the amide temperature coefficients had to be obtained from slices taken from Total Correlated Spectroscopy (TOCSY) spectra measured over the temperature range 285 to 312 K. The TOCSY spectra were acquired with 2048 points in $t_2$ and 256 points in $t_1$ with a mixing time of 70 ms. TOCSY FIDs were zero-filled to 1024 points in $t_1$ and apodized by multiplication with a sinebell squared function prior to Fourier transformation.

Two-dimensional Rotating frame nuclear Overhauser Enhancement Spectroscopy (ROESY) spectra were recorded at 298 K by the phase-sensitive TIPPI method with solvent suppression by selective saturation [14] on a Bruker Avance DRX 400 MHz spectrometer using a 5 mm multinuclear inverse probe equipped with xzy-gradient coils. ROESY spectra were acquired with 2048 points in $t_2$ and 256 points in $t_1$ with a mixing time of 250 ms. The FIDs were linear predicted in $t_2$ to 512 points, zero-filled to 1024 points and apodized by multiplication with a sinebell squared function prior to Fourier transformation. For the ROESY spectrum measured in aqueous solution, the residual water resonance was suppressed by time-domain deconvolution.

Diffusion coefficients were measured using a Bruker AM 500 MHz spectrometer specially modified to accommodate PFG experiments using the Longitudinal Eddy-Current Delay (LED) or Bipolar Pulse Longitudinal Eddy-Current Delay (BPPLED) pulse sequence [15, 16]. The intensity $I$ of the NMR signal in spectra measured with the BPPLED pulse sequence is related to the diffusion coefficient by the equation:

$$ I = I_o \exp\left[-D(\Delta - \delta - \tau/2)(g^2 - g_0^2)\right] $$

where $I_0$ is the intensity of the resonance in the NMR spectrum in the absence of external gradient pulses, $\Delta$ is the diffusion delay time, $\delta$ is the gradient pulse duration time, $\tau$ is a short delay following each gradient pulse, $g$ is the gyromagnetic ratio, $g_0$ is the gradient amplitude and $D$ is the self-diffusion coefficient. The equation for the LED pulse sequence is the same except that no $\tau/2$ term is needed because only one set of gradient pulses is applied. Typically, in each PFG-NMR experiment, a series of 15 to 35 LED or BPP-LED spectra were collected as a function of gradient amplitude. Duplicates were recorded for the peptide in TFE and in aqueous solution at 276.5 K and 289.3 K. The values of $\Delta$ and $\delta$ were held constant at 0.25 s or 0.4 s and 1 ms, respectively, while $g$ was varied from 0.4 to 8.5 Tm$^{-1}$. The value of $\tau$ was held constant at 1.2 ms. A delay, $T_{1e}$ of 20 ms at the end of the experiment was used to avoid spectral artifacts resulting from residual eddy currents. Diffusion coefficients of the peptide were calculated from the slope of the line obtained by plotting the natural logarithm of resonance intensity versus the square of the gradient amplitude. The line was determined using linear regression in Microcal Origin 4.10.

The viscosity of aqueous solutions was measured using an Ostwald viscometer purchased from the Cannon Instrument Company, State College, PA, USA. The temperature was controlled to ± 0.05 K with a VWR Scientific 1160 Temperature regulator. The viscosity of protonated water at 310 K was obtained from the literature [29] and the efflux time measured using a stopwatch. The viscosity ($\eta_2$) and the efflux time ($t_2$) for pure water at 310 K and the efflux time ($t_3$) for the 90\% H$_2$O/10\% D$_2$O solution at the appropriate temperature were used in the relationship:

$$ \frac{\eta_1}{\eta_2} = \frac{t_1}{t_2} $$

to obtain the viscosity ($\eta_1$) of the 90\% H$_2$O/10\% D$_2$O solution.

In order to obtain a more accurate value for the viscosity of $d_1$-TFE/D$_2$O solutions (which are known to be anomalous) than could be provided using the Ostwald method, microscopic viscosities were determined by measuring the diffusion coefficient for the residual protonated TFE in the $d_1$-TFE/D$_2$O solutions. The viscosity of protonated TFE obtained from the literature [18] was used to determine the viscosity of pure $d_1$-TFE using the Ostwald method as described previously. The viscosity of pure $d_1$-TFE ($\eta_1$) along with the diffusion coefficient of pure $d_1$-TFE ($D_1$) and the diffusion coefficient of $d_1$-TFE in the appropriate mixture ($D_2$) were used in the relationship $D_2/\eta_2 = D_1/\eta_1$ to calculate the viscosity($\eta_1$) of $d_1$-TFE in each mixture.

**Results and discussion**

The CD spectra of hGH(9–19) in a series of TFE/H$_2$O solutions at 298 K are shown in Fig. 1. The negative minima around 207 nm and 222 nm in 100\% TFE (f) are consistent with a helical conformation, with the greater intensity of the 207 nm peak indicative of a 3$_{10}$-helix [19]. The CD spectrum of the peptide in aqueous solution (a) is characteristic of random coil with negative minima around 200 nm [20]. Not surprisingly the peptide becomes increasingly helical as the amount of TFE is increased.

The proton NMR chemical shifts of the peptide in 90\% H$_2$O/10\% D$_2$O and in $d_1$-TFE at 298 K are listed in Table 1. Assignments were made using the sequential methodology [21]. Comparison of the measured alpha proton chemical shifts with the literature values for a random coil peptide (Table 2) provides additional qualitative information about the type of secondary structure present [22]. The upfield