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Determination of the relative NH proton lifetimes of the peptide analogue viomycin in aqueous solution by NMR-based diffusion measurement

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Abstract

In aqueous solution, exchanging peptide NH protons experience two environments, that of the peptide itself with a relatively slow diffusion coefficient and that of the water solvent with a faster diffusion coefficient. Although in slow exchange on the NMR chemical shift timescale, the magnetic field gradient dependence of the NH peak intensities in an experiment used to measure diffusion coefficients reflects the relative time periods spent in the two environments and this allows the determination of the relative solvent accessibility of exchangeable protons in peptides or proteins. To test this approach, the magnetic field gradient dependent intensities of the chemically shifted amide and amine NH protons of the peptide antibiotic viomycin have been measured using the high resolution longitudinal-eddy-current-delay (LED) NMR method incorporating solvent water peak elimination by non-excitation. The NH resonances of viomycin have been assigned previously and their relative exchange rates determined. Here, the gradient dependence of each NH proton intensity is reported, and these, after a bi-exponential least squares fitting, yield the fractional lifetimes of the protons spent in the peptide and water environments during the diffusion period of the experiment.

Introduction

One of the standard experiments conducted during NMR studies of protein structure is to measure the temperature dependence of NH proton chemical shifts (Wüthrich, 1986). Exchangeable protons with negligible temperature coefficients are regarded as having low accessibility to the solvent water. However, this requires the measurement of spectra at elevated temperatures with the consequent possibility of degradation or denaturation of the protein or peptide. The relative exchange rates of protons on NH groups can also be determined using exchange experiments with D_2O at a single temperature but this can produce deuteration of other exchangeable protons such as CH protons in histidinyl residues. Variations in pH of the sample also influence the exchange rate of solvent-accessible labile protons, but in addition extremes of pH may result in changes to peptide conformation or in denaturation of proteins. Consequently, an experiment which gives access to information on NH exchange in a non-invasive way is desirable and approaches include transfer-of-saturation experiments and two-dimensional NMR methods such as NOESY and EXSY (Wüthrich, 1986) and selective water resonance inversion (Grzesiek and Bax, 1993).

However, another possibility is to measure the apparent diffusion coefficients of the various NH protons as these will reflect the relative lifetimes of the protons on the peptide and on the solvent water. This

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approach has been tested using the exchange of protons between *N*-acetylaspartate and water (Moonen et al., 1992) and for proton exchange in a synthetic 16 base-pair DNA fragment (Böckmann and Guittet, 1997). Recently, a method based on a combination of a spin-echo diffusion sequence and a selective inversion ^{1}H-^{15}N HMQC experiment has been proposed and applied to water-amide exchange in acyl carrier protein (Andrec and Prestegard, 1997).

High resolution diffusion coefficient measurement experiments have become feasible following the development of the longitudinal-eddy-current-delay (LED) NMR pulse sequence (Gibbs and Johnson, 1991) which allows the determination of diffusion coefficients of molecules based on the attenuation behaviour of individual NMR resonances in the presence of magnetic field gradients. The LED sequence is a modification of the pulse-field gradient spin-echo experiment (Stilbs, 1987) which has long been the standard NMR spectroscopic method for measuring diffusion coefficients. A modified LED sequence incorporating a solvent suppression scheme, WATERGATE (Piotto et al., 1992) and bipolar gradients as this further reduces eddy current effects (Wu et al., 1995) has been used here. For non-exchanging protons, the diffusion coefficients are obtained by fitting the intensity of the NMR resonances as a function of the square of the applied gradient strength as originally shown by Stejskal and Tanner (1965). The LED sequence has been used extensively to obtain diffusion coefficients in a number of application areas including phospholipid vesicles (Hinton and Johnson, 1993), cell extracts (Barjat et al., 1995), polymer distributions (Chen et al., 1995) and biofluids (Liu et al., 1996, 1997). When the results are plotted as a pseudo-two-dimensional display with NMR chemical shifts on the horizontal axis and the derived diffusion coefficients on the vertical axis the method has been termed diffusion-ordered spectroscopy (DOSY) (Hinton and Johnson, 1993; Barjat et al., 1995; Chen et al., 1995). Extensions of the technique have been made by incorporating the basic LED sequence into two-dimensional NMR pulse sequences to produce pseudo-three-dimensional spectra such as DETOCSY (Liu et al., 1996, 1997), DOSY-COSY (Wu et al., 1996a), DOSY-HMQC (Lin and Shapiro, 1996), DOSY-HETCOR (Wu et al, 1996b) and DOSY-NOESY (Gozansky and Gorenstein, 1996). These produce reduced spectral overlap which allows improved determination of diffusion coefficients since the gradient dependence of the signal intensities are only monoexponential for a single peak.

The problems of measuring diffusion coefficients in the presence of chemical exchange which can occur in the diffusion period Δ in the LED sequence have also been addressed (Johnson, 1993). The NMR signals can be in fast, intermediate or slow exchange on the chemical shift timescale and all situations can be observed for the same molecule at any one temperature. Thus, consideration has to be given as to whether the spin system is in fast or slow exchange in terms of all NMR parameters including chemical shifts and relaxation times. The situation where a nucleus is in two environments but gives rise to only one chemical shift, i.e. fast exchange in chemical shift terms has been addressed specifically (Johnson, 1993). It has been shown that if diffusion is slow compared to the diffusion period, Δ, in the LED pulse sequence, then the gradient squared dependence of the peak intensity is bi-exponential and two diffusion coefficients result but, if diffusion is fast then a weighted average diffusion coefficient results. In the intermediate exchange situation, the analysis is more complicated. The situation where the chemical shifts of the exchanging species are in slow exchange, has also been considered (Lennon et al., 1994). Here, if the diffusion period is long enough for the exchange process to be in the fast exchange limit, then each of the sites will give separate chemical shifts and the apparent diffusion coefficients of the species at each site will be a weighted average of the diffusion coefficients of the species according to their relative populations. It has also been shown (Lennon et al., 1996) that it is important to take into account the relative relaxation times of the nuclei at the exchanging sites if this would result in some of the spectral intensity becoming 'NMR-invisible' as when binding to a macromolecule or a cell membrane.

The specific case of exchange between non-equivalent sites was first treated in a method named gradient-enhanced exchange spectroscopy (GEXSY) where the the apparent diffusion behaviour of exchangeable protons was explored using both one-dimensional and two-dimensional NMR experiments (Moonen et al., 1992). For intramolecular exchange, between for example water and a peptide, which is in the fast exchange limit during the diffusion period, the proton NMR cross-peak intensities show exponential behaviour according to Equation (1) (Stejskal and Tanner, 1965):

$$I_i / I_{0i} = \exp(-K^2 D_i \Delta) \qquad (1)$$

where I_{0i} is the intensity of peak *i* in the absence of any gradient, *K* is $\gamma_i G\delta$, where γ_i is the spin magnetogyric

ratio, G and δ are the strength and duration of the field gadient, and Δ is the diffusion period (Stejskal and Tanner, 1965). In the fast exchange limit, the observed D_i is the average of that for the water (D_w) and peptide environments (D_p) weighted by the relative lifetimes of the proton on water (f_w) and on the peptide (f_p). The probability that no exchange has taken place out of the peptide site is $\exp(-1/\tau_p)$ if τ_p is the lifetime of the proton in site p. Here we define $f_p = \tau_p/(\tau_p+\tau_w)$ as the fractional time that a proton stays in the p site during the diffusion time.

However, if the fast exchange limit is not obeyed, distinction needs to be made between spins which exchange during the diffusion period and those which do not. Thus outside the fast and slow exchange limits and when $\tau_p \ll \tau_w$, it has been shown (Moonen et al., 1992) that the gradient dependence is more complex and is given by Equation (2):

$$I_i = I_{0i}\{\exp(-K^2 D_w \Delta) - P\exp(-K^2 D_p \Delta)\}/ \{[1 - K^2(D_w - D_p) f_p \Delta][1 - P]\} \quad (2)$$

Viomycin, a cyclic peptide antibiotic which has been used in the treatment of tuberculosis, has been studied using NMR spectroscopy. The first study of NH exchange in viomycin in aqueous solution (Hawkes et al., 1978) derived full assignments and made studies of the NH solvent accessibility as a function of pH by the one-dimensional saturation transfer method. A later study (Dobson et al., 1986) using the two-dimensional NOE method also derived NH exchange rates. The molecular structure and numbering scheme for viomycin is shown in Figure 1 and this peptide has now been studied as a model system to investigate the feasibility of NMR diffusion measurements of apparent differential diffusion of NH protons as a measure of their accessibility to the solvent water. In the absence of ^{15}N labelled viomycin, a non-selective method based on ^{1}H NMR spectroscopy with non-excitation of the water resonance has been used. The initial study presented here is therefore used to demonstrate the feasibility of the use of relative diffusion coefficient measurements for investigating NH exchange in peptides and proteins.

Experimental methods

The sample of viomycin, dissolved in H_2O/D_2O 90/10 *v/v* at pH 5.6, a value which gives a well resolved

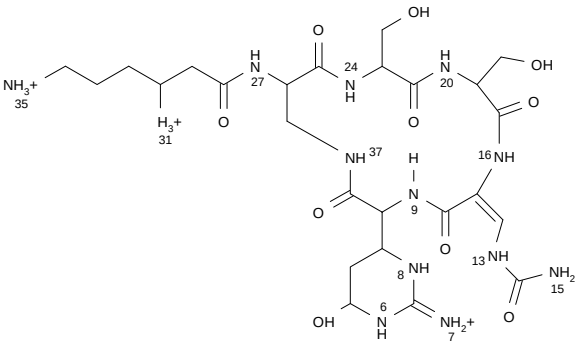

Figure 1. The structure and numbering system for viomycin.

NMR spectrum, at a concentration of 50 mM was obtained from Pfizer Inc. (New York, NY, USA).

All NMR spectra were measured on a Bruker AMX-600 spectrometer operating at 600.13 MHz for ^{1}H observation and using a Bruker B-AFPA30 gradient unit capable of providing gradients up to 2000 mT/m along the magnetic field direction. The data were acquired with a version of the LED delay pulse sequence (Gibbs and Johnson, 1991) with the inclusion of the WATERGATE solvent peak suppresion scheme (Piotto et al., 1992) to eliminate the resonance of the water without causing any transfer of saturation. A series of spectra were measured for values of the gradient strength in the range 20 mT.m^{-1} – 700 mT.m^{-1} in random order in steps of 20 mT.m^{-1} using bipolar sine-shaped gradients of base length 2 ms with a diffusion period of 300 ms. Typically, 128 transients were acquired into 32 768 data points over a spectral width of 7204 Hz at a temperature of 303 K. These data were multiplied by an exponential apodisation function corresponding to a line-broadening factor of 1 Hz and zero-filled by a factor of two prior to Fourier transformation. The intensities of the NMR signals were obtained using the standard Bruker peak picking software and were used to derive the lifetimes of a given proton on the peptide.

Determination of NH proton life times

The expression P in Equation (2) is equal to $\exp(-\Delta/f_p)$ where f_p is the fractional lifetime of the exchangeable hydrogen on the peptide during the diffusion period which is equal to $(\Delta - 5\delta/16 - \tau/2)$ for sine-shaped gradients. Δ is the time between the leading edges of the encoding and decoding gradients, δ is the gradient duration and τ is the time between the

two bipolar gradient components. Thus Equation (2) can be reformulated as

$$I_i = A_{0i}\{\exp(-K^2 D_w \Delta) - P\exp(-K^2 D_p \Delta)\} \quad (3)$$

where

$$A_{0i} = I_{0i}/\{[1 - K^2(D_w - D_p) f_p \Delta][1 - P]\}$$

The pulse sequence used here was a non-selective one-dimensional experiment and, unlike in the case of the two-dimensional GEXSY approach (Moonen et al., 1992), it is not possible to separate the exchanged part of an NH peak from the non-exchanged part during the diffusion period. Thus the observed intensity of an NH resonance in the diffusion measurement NMR experiment used here will comprise two components, an exchanged (*E*) part which transferred from water during the diffusion period and a non-exchanged (*N*) part, coresponding to the cross-peak and diagonal peak respectively of the GEXSY experiment. Based on Equation (2), in the fast exchange limit, the *E* and *N* components are affected by the magnetic field gradients according to Equations (4) and (5) respectively.

$$I(E)_i = I(E)_{0i}\exp\{[-K^2(D_w f_w + D_p f_p)\Delta]\} \quad (4)$$

$$I(N)_i = I(N)_{0i}\exp\{-K^2 D_p \Delta\} \quad (5)$$

Thus, since the components are not separated in the present work, the total intensity attenuation is bi-exponential.

$$I_i = I(E)_{0i}\exp\{[-K^2(D_w f_w + D_p f_p)\Delta]\} + I(N)_{0i}\exp\{-K^2 D_p \Delta\} \quad (6)$$

The observed NH resonance intensities were fitted to equation (6) to yield the relative lifetimes. In all cases a good fit was obtained using only the bi-exponential equation (6).

The diffusion coefficient of viomycin was obtained by averaging values based on twelve CH NMR resonances and that from water was taken from the water resonance intensity. The results were normalised to the known diffusion coefficient of water at 2.30×10^{-9} $m^2.s^{-1}$ (Hrovat and Wade, 1981).

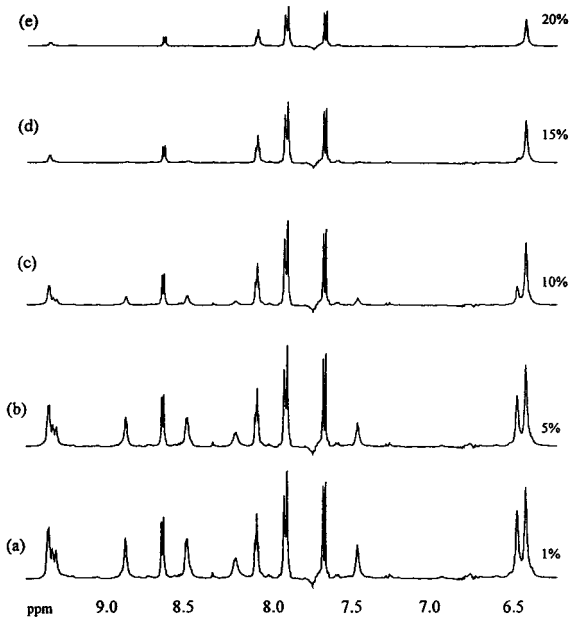

Figure 2. The NH region (δ6.2 – δ9.5) of the 600 MHz ^{1}H NMR spectrum of viomycin in H_2O/D_2O, 90/10 *v/v* at various gradient strengths using the LED pulse sequence with WATERGATE non-excitation of the water resonance. The assignments are given in Table 1, (a) 20 $mT.m^{-1}$ (b) 100 $mT.m^{-1}$ (c) 200 $mT.m^{-1}$ (d) 300 $mT.m^{-1}$ and (e) 400 $mT.m^{-1}$.

Results

The 600 MHz ^{1}H NMR spectra of viomycin in H_2O/D_2O shown in Figure 2 were obtained using the modified LED pulse sequence for diffusion coefficient measurement. The spectrum given in Figure 2a shows the spectrum obtained with a very low gradient value of 20 $mT.m^{-1}$ and this is essentially identical to a conventional spectrum. This shows only the region of the spectrum which includes all of the NH resonances and the olefinic CH resonance. The CH resonance which appears at δ7.9 is complicated by the fact that it comprises a doublet from molecules with a CH.NH moiety and an overlapping singlet from viomycin molecules containing a CH.ND moiety in slow exchange with each other. The assignment of the NH resonances has been achieved previously (Hawkes et al., 1978) and is as given in Table 1 according to the scheme shown in Figure 1.

Figures 2b-e show the same region of the spectrum but acquired with increasing values of the strength of the bipolar z-direction field gradient. All of the NMR resonances are diminished in intensity as the gradient strength is increased. However, the effects are not con-

Table 1. Chemical shifts and NH lifetimes of hydrogens in viomycin during the diffusion period

Assignment	Chemical shift (δ)	Lifetime on the peptide (ms)
NH(20)	9.36	102
NH(13)	9.31	60
NH(16)	8.88	57
NH(24)	8.66	120
NH(27)	8.50	66
NH(6)	8.19	0
NH(37)	8.07	129
CH	7.89	300
NH(9)	7.65	247
NH(8)	7.44	33
NH_2^+(7)	6.45	90
NH_2(15)	6.39	126
H_2O	4.67	–

stant for all of the peaks, and those in fastest exchange with the water are attenuated first. As can be seen from Figure 2, the NH resonances from the NH_3^+(31,35) groups do not appear in the spectrum using only a 20 mT.m^{-1} gradient and therefore these are the groups in fastest exchange with the water. From a qualitative inspection of the spectra, the next fastest to exchange is NH(6) from the six-membered ring and this has been virtually eliminated from the spectrum using a gradient level of 10%. There are four NH groups which are next most susceptible to loss of intensity and these are NH(16), NH(27), NH(8) and NH_2^+(7). The next fastest proton in order of exchange rate is NH(13) and somewhat slower is NH(20). The signal from NH(24) attenuates at higher gradient strength and then there is a final group where the attenuation caused by the gradient is considerably less. These protons therefore have less solvent accessibility and of this group, NH(37) and NH_2(15) lose intensity somewhat faster than the others. Three proton signals decrease in intensity least as a function of the gradient and these are the two signals from the =CH group and that from the NH(9) group. The attenuation of the =CH signals is representative of the overall diffusion of the whole molecule as this proton is not in exchange with the solvent water. The NH(9) proton has a gradient-related attenuation which is very similar to that of the overall molecule and thus this NH group has very little solvent accessibility on the timescale of the diffusion period consistent with it being part of an intramolecular hydrogen bond as found previously (Hawkes et al.; 1978, Dobson et al., 1986; Bycroft, 1972). In addition, the signals from NH(37) and NH_2(15) also show slow diffusion characteristics and therefore these groups must also have considerably restricted access to the solvent.

The qualitative results described above have been refined by calculation of the proton lifetimes on the peptide based on the various viomycin CH and NH resonances. The diffusion coefficient of viomycin was obtained as the mean value of 12 resonances in the aliphatic region of the spectrum with the value 2.73×10^{-10}m^2.s^{-1} fitted using the single exponential function of Equation (1). This is in the same range as but, as expected, smaller than the value for *N*-acetylaspartate (Moonen et al., 1992). Since there is a large concentration excess of water over peptide, the diffusion coefficient of water can be considered as independent of the exchange process and this was obtained from a separate experiment without solvent suppression and provided a value of 2.30×10^{-9}m^2.s^{-1}. The dependence of selected NH peak intensities on K^2 are shown in Figure 3, and it can be seen from the log plot that the behaviour is not simply exponential. The NH proton peak intensities were fitted to Equation (6) as described above to yield values for f_p the lifetime of the proton on the peptide, using values of D_p and D_w above. The derived values are given in Table 1.

Thus based on diffusion measurement, the relative NH lifetimes are NH(9) $\gg$ NH(15) $\approx$ NH(37) $\approx$ NH(24) $>$ NH(20) $>$ NH_2^+(7) $>$ NH(27) $\approx$ NH(13) $\approx$ NH(16) $>$ NH(8) $\approx$ NH(6) and finally the NH_3^+ groups. This order is in good agreement with previous work based on pH variation and saturation transfer experiments (Hawkes et al., 1978) and is also consistent with a study based on two-dimensional NOESY experiments when quantitative values for exchange rate constants (in s^{-1}) were obtained for some protons viz., NH(8), 1.45 $>$ NH(16), 1.21, NH(13), 1.18 $>$ NH(27), 0.75 $>$ NH_2(15), 0.52 $>$ NH(20), 0.44 $>$ $NH_2{}^+$(7), 0.39 $>$ NH(24), 0.13, but values for NH(16) and NH(37) could not be determined (Dobson et al., 1986).

Discussion and conclusions

The experiments described here have allowed the magnetic field gradient dependence of the various protons of viomycin to be determined using the LED diffusion measurement method. Assuming that the differential relaxation of a proton in the two sites does not in-

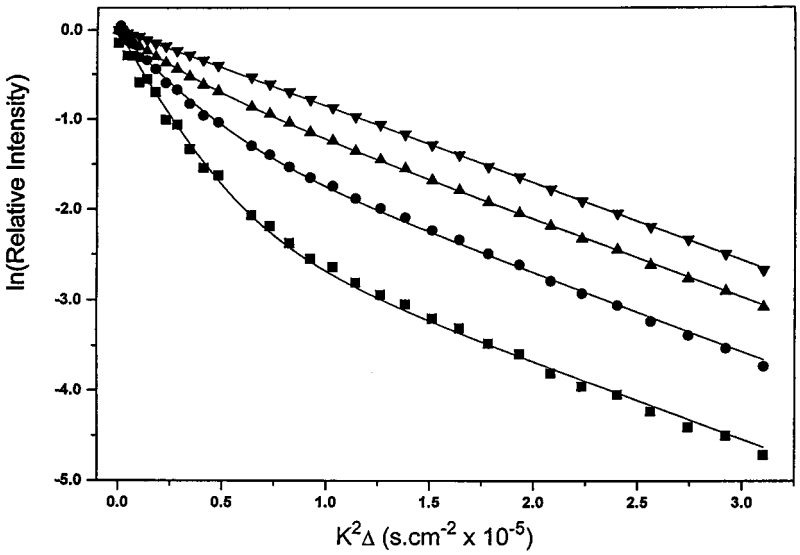

Figure 3. The log of the intensity of the NH NMR peaks from NH(20) ■, NH(24) ●, NH(37) ▲, and a viomycin aliphatic resonance (CH) ▼, as a function of K^2.

terfere with the interpretation (Lennon et al., 1996), then the observed apparent diffusion behaviour of the NH resonances reflects the relative lifetimes of the NH protons on the viomycin and the water. This provides a rapid method for studying NH exchange rates without the need for D_2O addition or experiments at elevated temperatures. The method can easily be extended to a three-dimensional version with diffusion coefficient on the third axis, such as a ^1H–^{15}N HMQC or HSQC experiment, as similar methods have already been achieved for other systems (Liu et al., 1996, 1997; Wu et al., 1996a, b; Lin and Shapiro, 1996; Gozansky and Gorenstein, 1996).

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