Journal of Biomolecular NMR, 5 (1995) 59–66
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J-Bio NMR 267

Determination of the rotational dynamics and pH dependence of the hydrogen exchange rates of the arginine guanidino group using NMR spectroscopy

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Received 30 December 1994
Accepted 20 January 1995

Keywords: Arginine; Hydrogen exchange; Side chain; Guanidino group; Dynamics; Flip rates

Summary

The dynamic behaviour of the guanidino group of arginine has been investigated quantitatively with the intention of providing a set of basis values for the interpretation of data acquired for arginine residues in proteins. At room temperature, a single broad resonance line is observed for the four η-NH_2 protons. Upon cooling the sample (≈ 10 °C at 500 MHz), two η-NH_2 proton resonances are resolved which were shown by HMQC spectroscopy to be the result of slowed rotation about the N_ε-C_ζ partial double bond. The flip rate (k_{NC}) about the N_ε-C_ζ bond was measured as a function of temperature using line-shape analysis of both ^1H and ^{15}N NMR spectra; at 25 °C, k_{NC} is between 900 and 1000 s^{-1}. The exchange broadening, due to N_ε-C_ζ bond flips, typically results in weak or missing signals for the η-NH_2 protons of arginine residues in HMQC or INEPT experiments recorded at room temperature, unless the motion is restricted in some way. In a related series of experiments, the pH dependence of the hydrogen exchange rates of the ε-NH and η-NH_2 protons of arginine was measured using saturation transfer ^1H NMR spectroscopy and compared with the equivalent NH_2 protons of the guanidinium ion. As expected, OH^- ion catalysis dominates over most of the pH range and proceeds at a rate close to the diffusion limit for both types of proton ($k_{OH} = 2 \times 10^9 - 1 \times 10^{10}$ $M^{-1}s^{-1}$, depending on conditions). At low pH values, however, catalysis by H_3O^+ becomes important and leads to characteristic rate minima in the exchange versus pH profiles. Acid catalysis is significantly more effective for the η-NH_2 protons than for the ε-NH proton; at low ionic strength (50 mM KCl) the rate minima occurred at pH 3.6 and 2.3, respectively. Under these conditions, acid-catalysed rate constants (k_H) of 706 $M^{-1}s^{-1}$ (η-NH_2) and 3 $M^{-1}s^{-1}$ (ε-NH) were obtained at 25 °C. At high ionic strength (1 M KCl) the rate of OH^- ion catalysis is decreased slightly, whereas the H_3O^+-catalysed rate is unchanged. The k_{OH} value of the free guanidinium ion is identical to that of the η-NH_2 protons but acid catalysis occurs less easily, leading to a rate minimum at pH 3.3.

Introduction

Arginine plays a special role in proteins, participating both structurally, as in the formation of stabilising salt bridges, and functionally, for example at substrate binding sites and interfaces. The large guanidino group of arginine is ideally suited to these activities; it bears a single positive charge, it is very difficult to deprotonate and the five nitrogen-bonded protons are capable of undergoing extensive hydrogen bonding interactions. Arginine is often found at the recognition sites for phosphate-containing substrates and cofactors (Riordan et al., 1977) and has also been proposed to participate in phosphoryl transfer reactions (Cotton et al., 1977; Knowles,

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Abbreviations: HMQC, heteronuclear multiple-quantum coherence; DEPT, distortionless enhancement by polarisation transfer; HSQC, heteronuclear single-quantum coherence; INEPT, insensitive nucleus enhancement by polarisation transfer; ppm, parts per million; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time.

1980). In nucleic acid binding proteins, notably zinc fingers, arginine can form both sequence-independent ionic interactions with the phosphate backbone and the critical hydrogen bonds with bases that contribute to sequence-specific binding (Pavletich and Pabo, 1991). Although its high pK_a precludes a catalytic role under most circumstances, arginine residues in several membrane-bound proton translocases (e.g., lac permease, ATP synthase, bacteriorhodopsin) have been implicated as participants in the proton translocation pathway (Menick et al., 1987; Senior, 1990; Balashov et al., 1993). In these cases, depression of the pK_a in the hydrophobic environment of the membrane is proposed to enable protonation and deprotonation of the guanidinium group.

The guanidinium group of protonated arginine is highly symmetric and very high pH values are required to form the free base ($pK_a = 12.5$ at 25 °C; Schmidt et al., 1930). High resolution X-ray and neutron diffraction studies of crystals of the free amino acid show the N_ε-C_ζ and two C_ζ-N_η bond lengths to be almost equal (1.33–1.34 Å), implying that they possess a similar degree of double-bond character (Karle and Karle, 1964; Lehmann et al., 1973). The N-C-N bond angles are close to 120° and the guanidinium group is planar, although a small deviation in planarity, to accommodate hydrogen bond formation, was noted for H_ε in the neutron diffraction study (Lehmann et al., 1973).

Early NMR experiments with arginine focused on the ^{15}N nucleus, which was used to monitor anion complexation, measure coupling constants, determine pK_a values and provide a qualitative picture of hydrogen exchange rates (Blomberg et al., 1978; Kanamori et al., 1978; Yavari and Roberts, 1978). The resolving power of the more recent ^{15}N-edited two- and three-dimensional techniques such as HMQC (Bax et al., 1983), HSQC (Bodenhausen and Ruben, 1980) and their derivatives, have made the protonated nitrogen atoms of individual amino acid side chains readily accessible (Muchmore et al., 1990) but in practice, attention is usually focused on the backbone amides as these are useful in structure determination. Nevertheless, a significant amount of data on the appearance of arginine side chains has accumulated in the literature and a quantitative basis for its interpretation is necessary. This paper addresses the flip rates about the partial double bonds of the guanidino group of arginine (free amino acid) and the hydrogen exchange rates of the guanidino protons as a function of pH.

Materials and Methods

Sample preparation

L-arginine was obtained from Sigma Chemical Company (St. Louis, MO) and was used without further purification. Guanidine hydrochloride (Ultrapure) was from ICN (Cleveland, OH). Samples used for measurement of guanidino group flip rates contained 30% methanol (methanol-d_3 in ^1H samples) to prevent freezing at the subzero temperatures required for conditions of slow exchange. Before the addition of methanol, the pH was adjusted to 3.7 at room temperature (the pH of minimum hydrogen exchange rate for the η-protons). Hydrogen exchange measurements were performed on 10 mM arginine (or guanidine) samples containing either 50 mM or 1 M KCl and 10% D_2O for lock purposes. pH adjustments were made by addition of small amounts of concentrated acid or base after removal of the sample from the NMR tube. A stock sample (1 cm^3 total volume) was maintained for these purposes, 0.5 cm^3 of which was returned to the NMR tube after each pH adjustment in order to keep the sample volume constant. The final pH was measured in the NMR tube directly after collecting the spectrum.

NMR spectroscopy

Spectra were recorded on Varian Unity 500 (^1H) and Varian Unity 300 (^{15}N) NMR spectrometers at 500 and 30.4 MHz, respectively. Both spectrometers were fitted with computer-controlled variable temperature units and external cryocool units for low-temperature work. Samples were allowed to equilibrate to the probe temperature for 30 min in the case of ^1H (0.5 cm^3 sample volume) or 1 h for ^{15}N (3.0 cm^3 sample volume). ^1H spectra were normally acquired using presaturation to suppress the solvent signal, as described in the appropriate figure legends. In the hydrogen exchange experiments, a parallel series of spectra were acquired using the 1–1 pulse sequence (Hore, 1983) to ensure that saturation transfer was the only process contributing to the change in intensity of the arginine guanidino protons. T_1 values were measured using a non-selective inversion recovery sequence with a 15 s relaxation delay. The recovery curves, which were always exponential, were fitted to a three-parameter function to account for imperfections in the 180° pulse. ^{15}N spectra were recorded with a pulse width of about 70°, a 1 s acquisition time and no delay between transients. WALTZ decoupling was applied throughout and the decoupler power was adjusted carefully to avoid unnecessary sample heating. ^1H spectra are referenced with respect to the methyl protons of sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) and ^{15}N spectra to 1 M NH_4Cl in 2 M HCl (external standard), adjusted to a liquid ammonia reference frequency according to Srinivasan and Lichter (1977).

Data analysis

Two-site exchange and hydrogen exchange data were analysed on a Sun workstation using an iterative nonlinear least-squares fitting program (CRVFIT), written and developed in-house. Flip rates about the N_ε-C_ζ partial double bond of arginine were determined from ^{15}N and

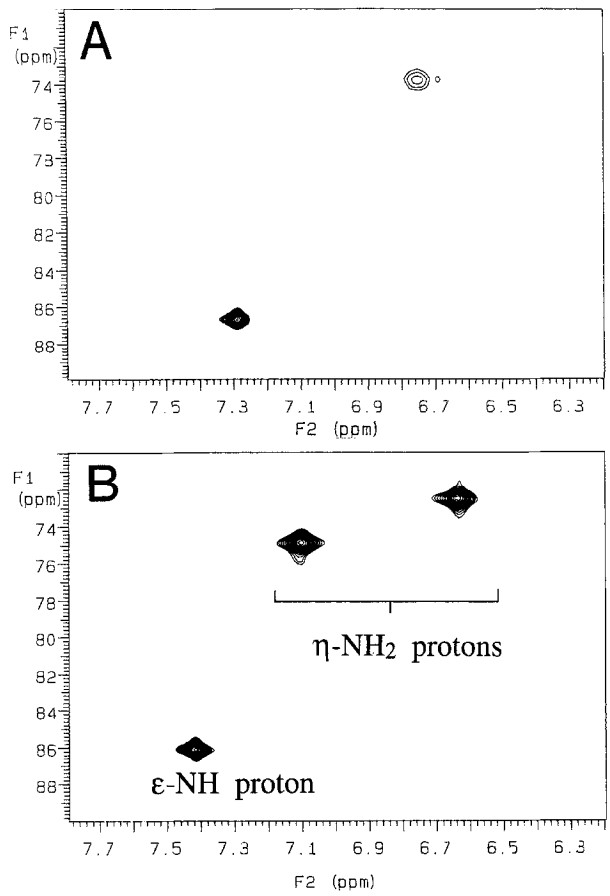


Fig. 1. 500 MHz (^{1}H)/50.7 MHz (^{15}N) natural abundance HMQC spectra of 200 mM arginine in 30% methanol-d_3 at (A) 35 °C and (B) −10 °C. The pH was adjusted to 3.7 (the pH of minimum hydrogen exchange rate for the η-protons) before addition of methanol. Spectra were collected with 1024 real data points in t_2 and 114 increments in t_1, with ^{15}N decoupling applied during the acquisition period. Per increment, 256 transients were collected and a weak solvent suppression pulse was applied during the relaxation delay (1.4 s). The ^{1}H (observe) and ^{15}N spectral widths were 6000 and 1500 Hz, respectively, resulting in a digital resolution of 0.26 ppm for ^{15}N. The data were zero-filled to 2048 (^{1}H) and 1024 (^{15}N) data points before processing. $^1J_{NH}$ was set to 90 Hz.

^{1}H NMR spectra, respectively, according to the standard two-site exchange equations of McConnell (1958). The pK_a values of water used in the calculation of hydrogen exchange rates ($pK_{a1} = -1.74$, $pK_{a2} = 15.7$ at 25 °C) were taken from Englander and Kallenbach (1984).

Results and Discussion

Rotational dynamics of the guanidino group

Qualitative information on the flip rates about the partial double bonds of arginine was obtained from natural abundance ^{1}H[^{15}N] HMQC spectra. The arginine sample used in these experiments contained 30% methanol to allow cooling below 0 °C and the pH was adjusted to 3.7 (measured at 25 °C), which is close to the pH of minimum hydrogen exchange rate for the η-protons (see below). All of the experiments described in this section are well within the slow limit for hydrogen exchange on the chemical shift time scale (i.e., hydrogen exchange does not affect the line shape), although transfer of saturation from the solvent occurs at higher temperatures.

Assuming complete rigidity of the arginine guanidino group, individual resonance lines are to be expected for each of the five guanidino protons and three guanidino nitrogens, as these are all magnetically inequivalent. In practice, only two peaks are observed in a ^{1}H[^{15}N] HMQC spectrum at 35 °C, one arising from the ε-proton and its coupled nitrogen and the other from all four equivalent η-protons coupled to the two equivalent η-nitrogen atoms (Fig. 1A). The presence of this single η-atom resonance suggests that the flip rates about the N_ε-C_ζ and C_ζ-N_η partial double bonds are all fast on the chemical shift time scale. At lower temperatures (−10 °C) the η-protons and η-nitrogens resolve into two distinct peaks (Fig. 1B), separated by 0.48 ppm in the ^{1}H dimension and 2.39 ppm in the ^{15}N dimension. This correlation pattern shows that the ^{1}H spectrum is responding to rotation about the N_ε-C_ζ bond, not about the C_ζ-N_η bond. The observation of two rather than four η-NH_2 proton resonances in the HMQC spectrum of arginine at −10 °C can be explained in two different ways: either the flip rate about the N_ζ-C_η partial double bonds is significantly higher than that of the N_ε-C_ζ bond, resulting in fast exchange conditions at −10 °C, or the chemical shift difference between the η_{11} (or η_{22}) and η_{12} (or η_{21}) protons is very small, so that a comparable flip rate leads to the appearance of fast exchange conditions.

The HMQC experiments demonstrate that the N_ε-C_ζ bond rotation in arginine is conveniently poised with respect to the NMR time scale and that analysis of either the ^{1}H or the ^{15}N line shapes can be used to determine the rate of this rotation. Representative ^{1}H spectra of the arginine guanidino group at various temperatures are shown in Fig. 2A. The mean lifetime, τ, can be extracted from the standard two-site exchange equations (McConnell, 1958) given the chemical shift separation, the line width in the absence of exchange and the fractional occupancy of each site. Under these conditions:

$$\tau = \tau_A \, \tau_B / (\tau_A + \tau_B) \quad (1)$$

where τ_A and τ_B are the lifetimes of a nucleus in sites A and B, respectively. When the fractional site occupancy is equal, as in this case:

$$\tau = 1/2\,k \quad (2)$$

where k represents the flip rate, k_{NC} (see, for example, Jardetzky and Roberts, 1981).

At higher temperatures, the intensities of both the η-NH_2 and ε-NH resonances are attenuated by transfer of saturation from water. Although the proton exchange rate

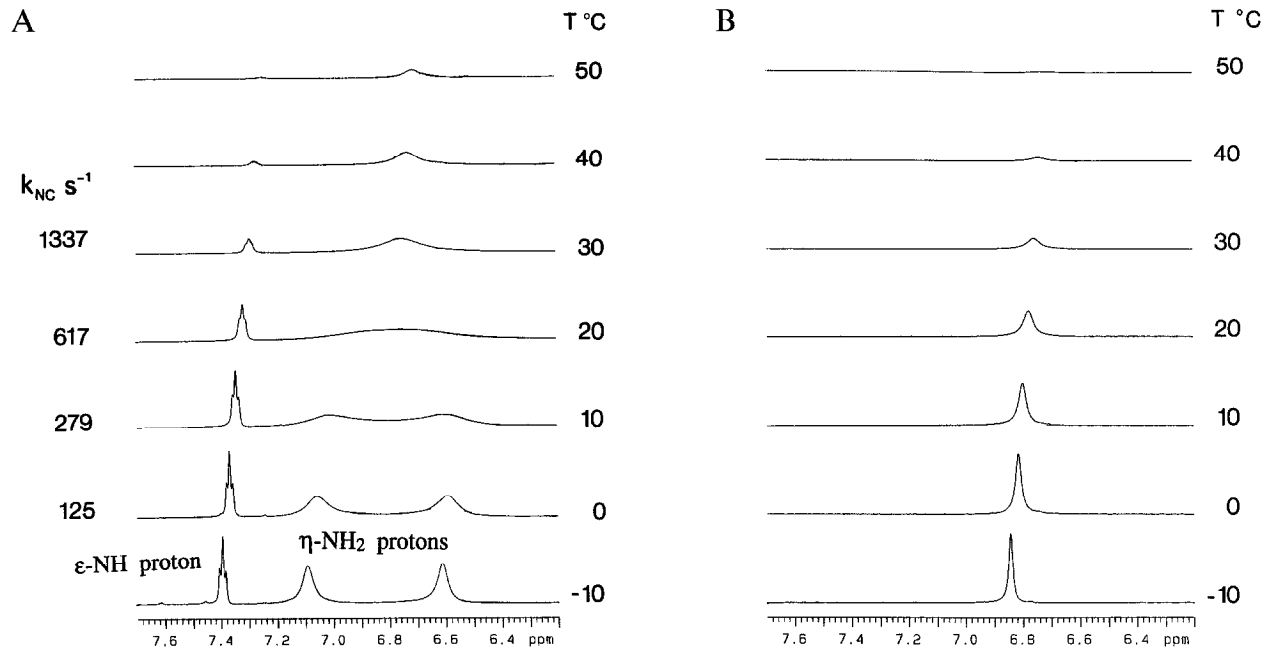


Fig. 2. Downfield region of the 500 MHz ^{1}H NMR spectra of (A) arginine and (B) guanidine in 70% water / 30% methanol-d_3 as a function of temperature. The pH was adjusted to 3.7 (close to the pH of minimum hydrogen exchange rate for the η-NH_2 protons) at 25 °C before the addition of methanol. Spectra were collected with a 3 s relaxation delay and solvent protons were suppressed by presaturation. At higher temperatures, the signal intensity is reduced due to transfer of saturation from the solvent. The effects of scalar relaxation with the coupled ^{14}N nucleus are apparent in the line widths of the ε-NH and guanidine protons. Fitted exchange rates are shown to the left of the spectra; the activation energy (54 kJ mol^{-1}) was obtained from seven spectra (not all shown) collected between 0 and 30 °C at 5° intervals. The Arrhenius factor A was 3.1×10^{12} s^{-1}.

is not fast enough to cause line broadening (scalar relaxation of the first kind; see below), the line width is affected by scalar coupling to ^{14}N (scalar relaxation of the second kind) which complicates the analysis. When scalar coupling dominates T_2, the ^{1}H line width increases with temperature because a decrease in correlation time decreases the efficiency of quadrupolar relaxation, which in turn results in less effective 'decoupling' from the observed proton. The dramatic effect of scalar coupling to ^{14}N (spin 1) can be seen in the temperature dependence of the spectrum of the parent molecule, guanidine (Fig. 2B). The guanidinium ion is completely symmetric and always exhibits a single ^{1}H resonance line; the line width varies from 7 Hz at −10 °C to 50 Hz at 60 °C. The ε-NH proton of the arginine also shows a significant, but smaller effect.

The spectra in Fig. 2 imply that the η-NH_2 proton line width measured under conditions of slow exchange is expected to be considerably narrower than the line width in the absence of exchange at higher temperatures, and is consequently unsuitable for determination of the N_ε-C_ζ flip rate. An approximate compensation for the temperature effects of scalar coupling was obtained from a comparison of the line width in the limit of fast exchange (28 Hz, 60 °C) with that of the guanidinium ion line width measured at the same temperature (50 Hz). The ratio of these two values was then used to calculate the arginine line width throughout the temperature range. As arginine is approximately three times the size of the guanidinium ion, its correlation time can be expected to be significantly longer and the line widths appropriately narrower. In practice, the exchange lifetime τ is a sensitive function of the initial line width only as the fast and slow limits are approached; near the coalescence point, the effect of line width on line shape becomes negligible. Therefore, careful treatment of the data ensures that the effects of scalar coupling are not a major source of error. The calculated rates of rotation about the N_ε-C_ζ partial double bond (k_{NC}) as a function of temperature are shown in Fig. 2A. A value of 910 s^{-1} was measured at room temperature (25 °C). Using data obtained between 0 and 30 °C, the activation energy for N_ε-C_ζ bond rotation was calculated to be 54 kJ mol^{-1}.

The flip rate about the N_ε-C_ζ bond was measured independently using ^{15}N NMR at 30.4 MHz (Fig. 3). As relaxation of a protonated ^{15}N atom is dominated by dipolar coupling to the attached protons, the line width of the η-nitrogen atoms (two attached protons) should be twice that of the ε-nitrogen (one attached proton) in the absence of exchange. The latter value, which is easily measured experimentally, was used to calculate the line width of the η-nitrogen atoms at each temperature. Under these conditions (different nucleus, magnetic field strength and frequency separation in the absence of exchange), coalescence occurs at about 0 °C. The activation energy determined from ^{15}N NMR is 61 kJ mol^{-1} and the flip rate about the N_ε-C_ζ bond (k_{NC}) at 25 °C is calculated

to be 1000 s^{-1}, in reasonable agreement with the ^1H data. The major source of error in these experiments is the accuracy of the spectrometer temperature calibration, which probably accounts for most of the difference between the ^1H and ^{15}N experiments.

A brief survey of the literature suggests that the majority of the assigned arginine guanidino resonances in proteins resemble free arginine with respect to the flip rates about the partial double bonds of the guanidino group. This is not surprising, as arginine residues are frequently found on protein surfaces where the constraints on rotation about the partial double bonds are expected to be minimal. Under conditions of extensive hydrogen bonding, however, all five guanidino protons can be resolved in an HMQC spectrum at room temperature and above, as found for example for Arg^{75} in the Ca^{2+}-binding protein oncomodulin (Gagné, S.M. and Sykes, B.D., unpublished observations).

The major practical consequence of guanidino group flipping in proteins is that, although a strong resonance is usually observed for the ε-NH proton of arginine, the four η-NH_2 protons are frequently broad or missing

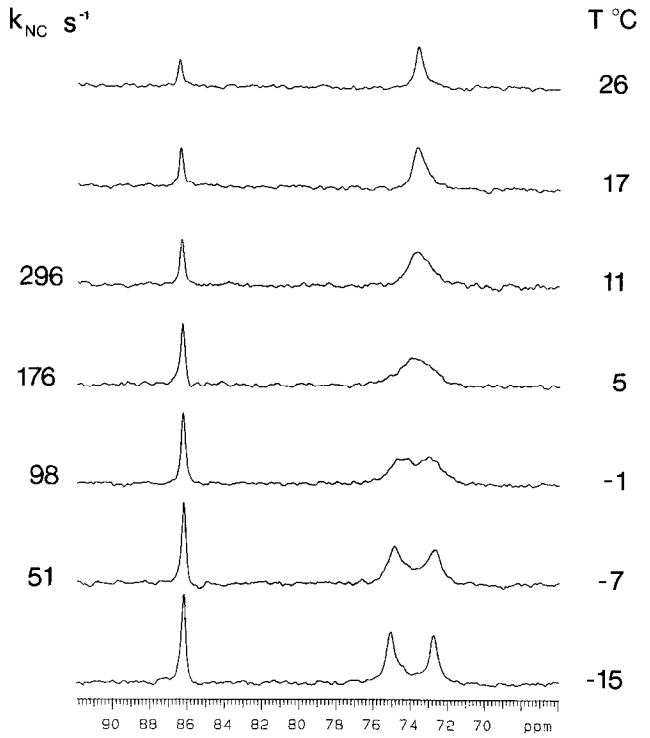


Fig. 3. 30.4 MHz ^{15}N NMR spectra of 200 mM arginine in 70% water / 30% methanol as a function of temperature. The pH was adjusted to 3.7 (close to the pH of minimum hydrogen exchange rate for the η-NH_2 protons) at 25 °C before addition of methanol. Per spectrum, 10 000 transients were averaged; other conditions are described in the Methods section. Fitted hydrogen exchange rates are shown to the left of the spectra. The activation energy (61 kJ mol^{-1}) was obtained from eight spectra (not all shown) collected between −7 and 14 °C at 3° increments. The Arrhenius factor A was 4.6×10^{13} s^{-1}. The reduction in signal intensity that occurs at higher temperatures is the result of the increase in T_1 that occurs as the solvent viscosity decreases.

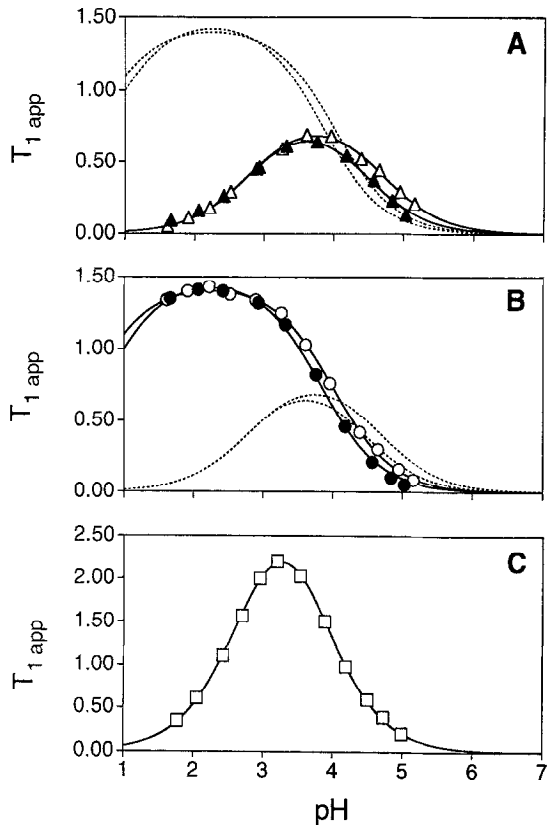


Fig. 4. Effect of saturation transfer on T_1 of arginine guanidino protons and guanidine at 25 °C as a function of pH. (A) η-NH_2 in 0.05 M KCl (▲) and η-NH_2 in 1 M KCl (△); (B) ε-NH in 0.05 M KCl (●) and ε-NH in 1 M KCl (○); (C) guanidine protons in 0.05 M KCl. Data were fitted to a combination of Eqs. 3 and 4 with the base-catalysed rate constant defined according to Eq. 5. The pK_a values were taken as 12.5 for arginine (both proton types) and 13.6 for guanidine (see text).

altogether at normal temperatures (20–30 °C). Differential rates of hydrogen exchange are often assumed to be responsible; however, these experiments demonstrate that this is typically not the case. DEPT-, HMQC- or INEPT-based experiments are particularly susceptible to loss of signal from the exchange-broadened η-NH_2 protons, because of the long ($1/J_{NH}$) focussing delays.

Hydrogen exchange rates of the guanidino protons

The exchange rates of the guanidino protons with solvent protons are expected to be fast in comparison with backbone amides (Blomberg et al., 1976; Wüthrich and Wagner, 1979, Englander and Kallenbach, 1984). OH^-, H_2O and H_3O^+ are all potential catalysts of hydrogen exchange, although buffer ions are unlikely to be effective catalysts because of the high arginine pK_a (see below). Exchange rates were determined at 25 °C in a saturation transfer experiment, in which apparent T_1 values (T_{1app}) were measured as a function of pH. Under these conditions the ε-NH proton is a moderately narrow triplet and the η-NH_2 protons appear as a single broad

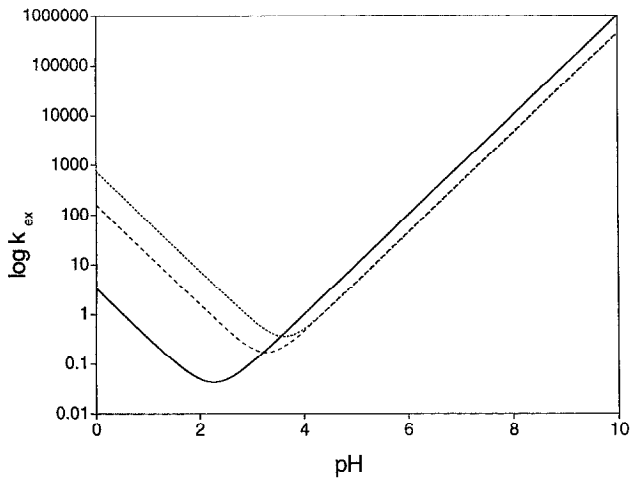


Fig. 5. Log k_{ex} versus pH profiles for arginine ε-NH (—), guanidine (---) and arginine η-NH_2 (····) protons calculated from the experimental k_{OH} and k_H values obtained at 50 mM KCl (Table 1).

line (see Fig. 2A). The apparent relaxation rate is given by:

$$1/T_{1app} = k_{ex} + 1/T_1 \quad (3)$$

where T_1 is the 'true' T_1 value in the absence of exchange and k_{ex} is the hydrogen exchange rate. In the limit of fast exchange, the measured relaxation rate $1/T_{1app}$ approaches k_{ex} and T_{1app} is consequently very short. When exchange is slow, on the other hand, the measured relaxation rate approaches the actual value $1/T_1$.

T_1 values obtained as a function of pH for both types of arginine proton are shown in Figs. 4A and B. Differences in the behaviour of the two types of proton are immediately apparent; acid catalysis dominates exchange of the η-protons below pH 3.6 in a manner reminiscent of a peptide amide, whereas acid-catalysed exchange is much less important for the ε-NH proton and does not contribute significantly until a much lower pH value (about 2.2). Although very little data were collected on the acid-catalysed limb of Fig. 4B, the shape of the curve was found to be highly reproducible. A small reduction of the base-catalysed rate, resulting in slightly longer relaxation times, was observed when the KCl concentration was increased from 50 mM to 1 M, although the acid-catalysed limb was unaffected. This is the expected result of increased screening of the positively charged guanidino group from the negatively charged OH^- catalyst. For comparison, the hydrogen exchange profile of the guanidinium ion in 50 mM KCl was also determined (Fig. 4C). As might be anticipated, the guanidinium ion exchange curve resembles that of the arginine η-NH_2 protons more closely than that of the ε-NH proton.

The hydrogen exchange rate, k_{ex}, is determined by the sum of the independent processes occurring for all catalytic species in solution. A significant contribution from pH-independent H_2O catalysis is to be expected only at very low pH values (Wüthrich and Wagner, 1979) and can be safely neglected in the presence of acid catalysis. The hydrogen exchange rate, k_{ex} is given by:

$$k_{ex} = k_H [H^+] + k_{OH} [OH^-] \quad (4)$$

where k_H and k_{OH} are the acid- and base-catalysed rate constants, respectively. Values for the catalytic rate constants were derived by fitting the T_{1app} data to Eq. 3 with k_{ex} defined according to Eq. 4. As the exchange rate never reaches the slow limit (although a close approach occurs for the ε-NH proton), the T_1 value in the absence of exchange cannot be measured and must be included in the fit. A unique fit for all three unknown parameters, k_H, k_{OH} and T_1, can be obtained if the pK_a for deprotonation of the guanidino group is taken into account for base catalysis, as described below.

Understanding the exchange behaviour of arginine requires knowledge of the principles of hydrogen exchange chemistry (see Englander and Kallenbach, 1984). Exchange occurs via transient hydrogen bond formation between a proton donor, AH, and an acceptor, B. When the complex dissociates, the fraction of protons remaining on A is determined by the relative pK_a values of AH and BH. The overall rate of proton transfer, k, is determined by the product of the fractional redistribution of protons and the number of collisions, k_D, which is assumed to be diffusion controlled:

$$k = k_D (10^{\Delta pK}/(10^{\Delta pK} + 1)) \quad (5)$$

where $\Delta pK = pK_a$ (acceptor) $- pK_a$ (donor) and k_D is the

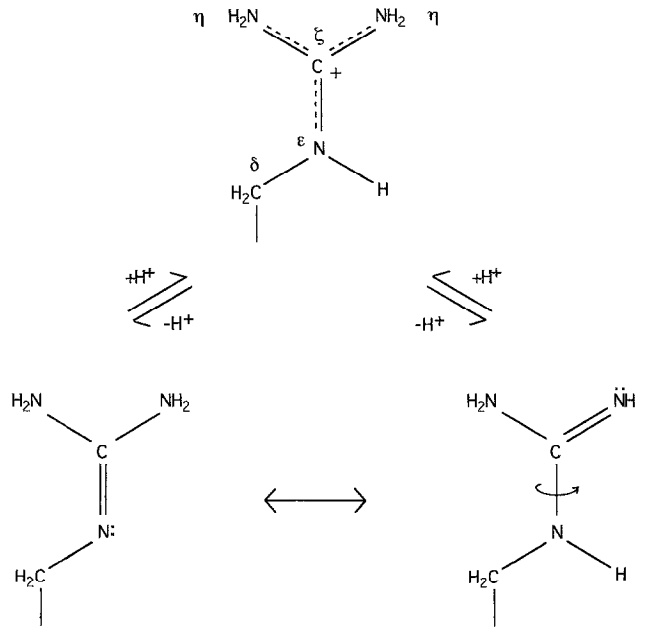
TABLE 1
HYDROGEN EXCHANGE RATE CONSTANTS OF THE ARGININE GUANIDINO GROUP AND GUANIDINE

Proton	pK_a	[KCl] (M)	k_{OH} (k_D) ($M^{-1}s^{-1}$)	k_H ($M^{-1}s^{-1}$)	T_1 (s)	pH_{min} [a]
Arginine ε-NH	12.5	0.05	$9.95 (\pm 0.47) \times 10^9$	3.3 (± 0.9)	1.52	2.26
Arginine η-NH_2	12.5	0.05	$4.37 (\pm 0.40) \times 10^9$	706 (± 7)	0.84	3.60
Arginine ε-NH	12.5	1.00	$6.73 (\pm 0.12) \times 10^9$	2.2 (± 0.5)	1.47	2.26
Arginine η-NH_2	12.5	1.00	$2.52 (\pm 0.21) \times 10^9$	757 (±36)	0.84	3.74
Guanidine	13.6	0.05	$4.50 (\pm 0.1) \times 10^9$	158 (± 3.6)	3.51	3.27

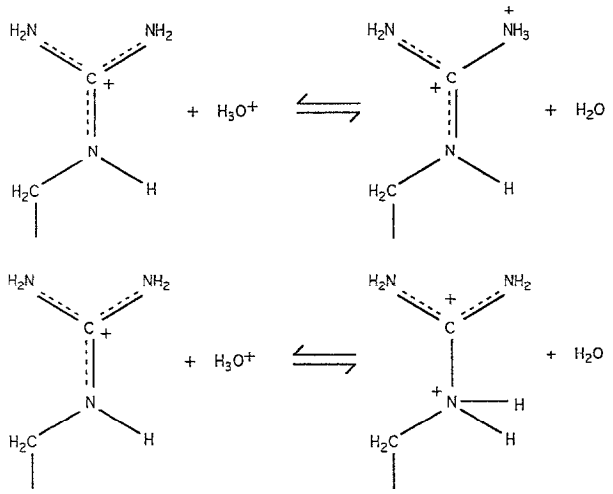
[a] Calculated from $pH_{min} = 0.5 [pK_w - \log (k_{OH}/k_H)]$.

second-order diffusion-limited rate constant (normally between 10^9 and 10^{11} $M^{-1}s^{-1}$; Eigen and Hammes, 1963). Removal of a proton from a side-chain nitrogen such as arginine ($pK_a = 12.5$; Schmidt et al., 1930) by OH^- is very efficient as the pK_a for protonation of OH^- is 15.7 and virtually every encounter succeeds (ΔpK is large and positive, and exchange proceeds at the diffusion-limited rate as $k = k_{OH} = k_D$). This behaviour is in complete contrast with that of the more familiar peptide amide ($pK_a \gg 15.7$), where the great majority of encounters are non-productive. By redefining k_{OH} in terms of Eq. 5, a unique fit can be obtained for the data in Fig. 3. These values are collected in Table 1 and the calculated rate versus pH curves are shown in Fig. 5. These are a set of basic curves to which arginine residues in proteins may be compared.

Almost identical k_{OH} values were obtained for guanidine and the arginine η-NH_2 protons, whereas the arginine ε-NH protons exchange twice as fast. An approximate twofold difference in the rates of base catalysis for the two types of arginine proton was noted previously by Yavari and Roberts (1978) using qualitative line-shape analysis of coupled ^{15}N spectra. Interpretation of the k_{OH} values in Table 1 requires consideration of some more subtle points. The predominant tautomeric form of neutral arginine is not known (see Scheme 1) and it is unlikely that both types of guanidino proton are equally easy to remove (differential proton affinities are observed, for example, in the acid-catalysed regime). The pK_a value of 12.5, therefore, can only be applied with certainty to the more labile proton. Provided the pK_a value for formation of the alternative tautomer is less than 15.7, this makes no difference to the extracted value for k_{OH}, because exchange occurs at the diffusion-limited rate and the



Scheme 1. Possible tautomeric forms of deprotonated arginine (neutral side chain).



Scheme 2. Transient protonation of arginine during acid-catalysed hydrogen exchange.

faster hydrogen exchange rate of the ε-NH proton can be explained in terms of factors that affect formation of the collision complex. The planar guanidino group is sterically crowded, with only 2 Å between the η_{12} and η_{21} protons (Lehmann et al., 1973), which may restrict the access of an incoming OH^- ion. Alternatively, the positive charge distribution within the guanidino group could influence the diffusion-limited rate for the different types of arginine proton. The possibility that the slightly slower rate obtained for the η-NH_2 protons may be caused by a high pK_a (≈ 15.7) associated with the minor tautomer cannot be rigorously excluded; however, it seems unlikely that this pK_a would be higher than that of guanidine (13.6). The close correspondence of the arginine η-NH_2 protons and those of the fully symmetrical guanidinium ion in the base-catalysed regime is good evidence for diffusion control. In this context it should be noted that the k_{OH} (k_D) values of arginine and guanidine are slightly lower than the diffusion-limited rate constant normally expected for oppositely charged species (Eigen and Hammes, 1963). In a parallel series of experiments, a more typical k_{OH} (k_D) value of 2×10^{10} $M^{-1}s^{-1}$ was obtained for the ε-amino group of a lysine derivative (Henry, G.D. and Sykes, B.D., unpublished data).

Equation 5 can also be used to explain the absence of water catalysis. Removal of a guanidino proton by water is expected to be very ineffective as the low pK_{a1} value of water (−1.74) makes ΔpK large and negative. Using Eqs. 4 and 5, a k_D value of 10^{10} $M^{-1}s^{-1}$ and a catalyst concentration of 110 M (i.e., assuming both water protons are independently capable of forming a collision complex), water catalysis can be calculated to occur at a constant rate of 6×10^{-3} s^{-1} throughout the pH range. This is much slower than the sum of the acid- and base-catalysed rates at all pH values and water catalysis should be insignificant (Fig. 5).

The occurrence of acid-catalysed hydrogen exchange in arginine was first noted by Blomberg et al. (1978) in an early ^{15}N study of amino acids. It implies that the guanidino group, which already possesses a positive charge, can accept a second proton. The probable mechanism of acid catalysis is shown in Scheme 2.

The k_H value reflects the ease of protonation, i.e., the η-nitrogens of arginine are slightly better proton acceptors than the guanidinium ion and significantly better than the arginine ε-nitrogen atom. Assuming a k_D value of 10^{10} $M^{-1}s^{-1}$, pK_a values of –8.9 and –9.5 can be calculated for arginine (η-nitrogen) and guanidine, respectively, using Eq. 5.

Conclusions

The appearance of the guanidino group of arginine is a sensitive function of the flip rate about the N_ε-C_ζ partial double bond. This quantitative study provides the necessary foundation for the interpretation of arginine side-chain cross peaks obtained in two- and three-dimensional spectra of ^{15}N-labeled proteins. As the effects of N_ε-C_ζ flipping are readily apparent over the temperature range normally encountered in biological NMR (5–40 °C), the appearance of arginine resonances should be a useful indicator of motional restriction on any given arginine residue. Motionally restricted residues may participate in the formation of salt bridges or in the hydrogen bonding networks responsible for maintaining the tertiary fold or mediating a conformational change. These are obviously of great interest from a functional point of view. Participation of arginine residues at a recognition site (e.g., for DNA or a substrate analogue) may be indicated by a change in motional restriction of the guanidino group upon addition of the molecule of interest. It should be noted that the experiments described here using free arginine are sensitive only to N_ε-C_ζ bond rotation; no information about C_ζ-N_η bond rotation rates could be obtained. In proteins, rotation about both bonds may be sufficiently slow for all five guanidino protons to be distinguished at room temperature. Measurement of the hydrogen exchange rates of the protonated guanidino nitrogen atoms reveals that acid catalysis makes a significant contribution to the rate versus pH profile of free arginine, particularly in the case of the η-NH_2 protons. In this respect, arginine resembles the peptide amide. In contrast to the peptide amide, however, the pK_a of arginine (12.5) ensures that base catalysis normally proceeds at a diffusion-limited rate. This is also likely to be the case for most arginine residues in proteins. Observation of a slowly exchanging arginine indicates that base catalysis is no longer diffusion-controlled; thus, the effective pK_a has been raised to approach or exceed that of OH^- (15.7 at 25 °C). Such a residue is likely to be of structural significance (e.g., salt bridge or hydrogen bonds) and hydrogen exchange rates should serve as a useful complement to flip rate measurements. Finally, it is important to note that in INEPT or HMQC spectra (or their derivatives) the absence of arginine η-NH_2 peaks, under conditions where the ε-NH peak is observed, is a feature of line broadening due to rotation about the N_ε-C_ζ partial double bond and is not a reflection of the relative hydrogen exchange rates of the two types of proton.

Acknowledgements

We thank Gerry McQuaid and Colin Bigam for continued maintenance of the NMR spectrometers, Robert Boyko for adapting his program CRVFIT to the two-site exchange and hydrogen exchange experiments and David Hoyt for providing the initial impetus for studying hydrogen exchange rates in side chains. This work was supported by the Medical Research Council of Canada.

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