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AMINE INVERSION IN PROTEINS

A ^{13}C-NMR STUDY OF PROTON EXCHANGE AND NITROGEN INVERSION RATES IN N^ϵ,N^ϵ,N^α,N^α-[^{13}C]TETRAMETHYLLYSINE, N^ϵ,N^ϵ,N^α,N^α-[^{13}C]TETRAMETHYLLYSINE METHYL ESTER, AND REDUCTIVELY METHYLATED CONCANAVALIN A

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Exchange rates were calculated as a function of pH from line widths of methylamine resonances in ^{13}C-NMR spectra of N^ϵ,N^ϵ,N^α,N^α-[^{13}C]tetramethyllysine (TML) and N^ϵ,N^ϵ,N^α,N^α-tetramethyllysine methyl ester (TMLME). The pH dependence of the dimethyl α-amine exchange rate could be adequately described by assuming base-catalyzed chemical exchange between two diastereotopic methyl populations related by nitrogen inversion. Deprotonation of the α-amine was assumed to occur by proton transfer to (1) OH^-, (2) water, (3) a deprotonated amine or (4) RCO_2^-. Microscopic rate constants characterizing each of these transfer processes (k_1, k_2, k_3 and k_4, respectively) were determined by fitting the rates calculated from line width analysis to a steady-state kinetic model. Using this procedure it was determined that for both TML and TMLME $k_2 \approx 1$–10 M^{-1} s^{-1}, $k_3 \approx 10^6$ M^{-1} s^{-1} and k_i, the rate constant for nitrogen inversion was about 10^8–10^9 s^{-1}. Upper limits of 10^{12} and 10^3 M^{-1} s^{-1} could be determined for k_1 and k_4, respectively. A similar kinetic analysis was used to explain pH-dependent line-broadening effects observed for the N-terminal dimethylalanyl resonance in ^{13}C-NMR spectra of concanavalin A, reductively methylated using 90% [^{13}C]formaldehyde. From exchange data below pH 4 it could be determined that amine inversion was limited by the proton transfer rate to the solvent, with a rate constant estimated at 20 M^{-1} s^{-1}. Above pH 4, exchange was limited by proton transfer to other titrating groups in the protein structure. Based upon their proximity, the carboxylate side chains of Asp-2 and Asp-218 appear to be likely candidates. The apparent first-order microscopic rate constant characterizing proton transfer to these groups was estimated to be about 1×10^4 s^{-1}. Rate constants characterizing nitrogen inversion (k_i), proton transfer to OH^- (k_1) and proton transfer to the solvent (k_2) were estimated to be of the same order of magnitude as those determined for the model compounds. On the basis of our results, it is proposed that chemical exchange processes associated with base-catalyzed nitrogen inversion may contribute to ^{15}N or ^{13}C spin-lattice relaxation times in reductively methylated peptides or proteins.

1. Introduction

Recently a new method for studying the environments of NH_2-terminal and lysyl ϵ-amino groups of proteins has been developed which utilizes ^{13}C-NMR of proteins reductively methylated with [^{13}C]formaldehyde and $NaCNBH_3$ [1–3]. The method has been shown to be of particular value for studying specific ion-pair interactions involving lysine side chains in hen egg lysozyme [4] and ribonuclease A [3,5]. We have recently applied the same reductive methylation procedure to concanavalin A (Con A), a plant lectin which, in its isolated form, relies upon the presence of bound Mn^{2+} and Ca^{2+} for its affinity for saccharides [6]. During the course of our study on the pH behavior of chemically modified groups in the protein, we noticed anomalous broadening of resonances in spectra acquired below pH 7. This effect was

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particularly evident in the case of the N-terminal dimethylalanyl resonance, where at pH 3, this resonance had broadened to the extent of becoming nearly nondetectable. Analogous broadening has also been observed in the case of the dimethyllysine resonances in ^{13}C-NMR spectra of lysozyme, where it was attributed to chemical shift nonequivalence of the diastereotopic methyl groups [5].

Historically, NMR techniques have been used to study the interconversion of nitrogen-containing alkyl cyclic and acyclic diastereomers at low temperatures and in aprotic solvents [7–14] in order to slow the exchange rates and minimize the contribution proton exchange makes to the lifetime of the exchanging species. Saunders and Yamada [15] were instrumental in demonstrating that nitrogen inversion in substituted benzyl amines could be studied in aqueous media under strongly acidic conditions, where base-catalyzed inversion was sufficiently slow so as to make characterization of the exchange processes feasible using NMR techniques. Since that time, it has been shown that nitrogen inversion and corresponding proton-exchange processes of alkyl amines in water can be determined by fitting pH-dependent NMR exchange data to a variety of kinetic models [16–21].

In order to characterize more fully the rate processes leading to the anomalous broadening effects observed for the dimethylamine resonances in our ^{13}C-NMR spectra of Con A, we have chosen to undertake first a study of two model compounds $N^{\epsilon},N^{\epsilon},N^{\alpha},N^{\alpha}$-[^{13}C]tetramethyllysine (TML) and its corresponding methyl ester (TMLME). These compounds offer the advantages of resembling modified protein structures, while having a limited number of deprotonation pathways, hence limiting the scope of any kinetic models posed. We show that the broadening of resonances observed in these compounds leads to a description of exchange processes which can be adequately modeled only by assuming inversion rates of the nonprotonated amine nitrogen of the order of 10^8–10^9 s^{-1}. We estimate that nitrogen inversion may be of importance in studies of the structure and dynamics of lysine residues of native or chemically modified proteins by ^{15}N- or ^{13}C-NMR.

2. Experimental

2.1. Materials and methods

[^{13}C]Paraformaldehyde, enriched to 90% abundance, was purchased from Cambridge Isotopes (Cambridge, MA). Conversion to the monomeric form was accomplished by heating a 10% aqueous suspension of the polymer to 110°C for 12 h in a sealed glass vial. All other chemicals were of reagent grade and were used without further purification. Con A was purified from jackbean meal by the affinity chromatography method as described by Agrawal and Goldstein [22], then purified of its fragmented subunits as described by Cunningham et al. [23].

Con A was reductively methylated according to the procedure of Jentoft and Dearborn [1] as described by Sherry and Teherani [6]. Purity of the protein preparation was checked by gel electrophoresis in the presence of 1% SDS.

Lysine and lysine methyl ester were reductively methylated by first preparing a 10 mM solution of these compounds containing 50 mM [^{13}C]formaldehyde, adjusted to pH 7 (10 ml total volume). Solid $NaCNBH_3$ was then added to a final concentration of 80 mM. Previous analytical studies have shown that under these experimental conditions, both amino groups will be completely dimethylated [6]. For solutions containing lysine, the reaction mixture was allowed to stand at room temperature overnight without further pH adjustments. For solutions containing lysine methyl ester, the pH of the reaction mixture was kept between 7 and 7.5 for 4 h by periodic addition of 0.1 M HCl. Following the reaction period, each of the reaction mixtures was adjusted to pH 2 using 1 M HCl and evaporated to dryness under reduced pressure. The pK_a values of the ϵ-dimethyl amino and α-dimethyl amino groups of these compounds were determined in water by potentiometric titration.

2.2. NMR spectra

Fourier-transformed ^{13}C-NMR spectra were acquired at 50.1 MHz (46.9 kG) on a JEOL FX-200 using broad-band proton decoupling. The samples were maintained at a probe temperature of 25°C using a flow of dry air. Spectra of the lysine derivatives were acquired using 10-mm NMR sample tubes with a coaxial insert containing methanol in ^2H_2O for the dual purpose of having an external reference and a field lock. pH titration of samples was carried out in the NMR tube using 1–5 M HCl or NaOH. Chemical shifts were measured digitally and are reported downfield of tetramethylsilane using methanol as an external reference standard in the case of the model compounds or as an internal reference standard (at 49.4 ppm) in the case of modified protein samples.

2.3. Computations

Exchange rates for TML and TMLME were determined by reading digitized NMR spectra into an iterative version of DNMR3, a computer program written for the calculation of complex exchange-broadened NMR spectra [24] (DNMR3IT_2 was obtained through Quantum Chemistry Program Exchange, Indiana University, Bloomington, IN). Populations of each of the two exchanging methyl groups were held fixed at mole fractions of 0.50, in agreement with measured peak intensities in spectra acquired at low pH. The chemical shifts of resonances arising from the two nonexchanging populations were estimated from the spectra at pH 2. Line widths in the absence of exchange were estimated from the exchange averaged resonance at high pH. Values obtained using this procedure (line widths of 2 Hz at limiting chemical shifts of 40.4 and 43.5 ppm) were treated as fixed parameters in the fitting routine.

Microscopic rate constants were obtained by fitting the pH dependence of NMR exchange rates to the kinetic model proposed by Delpuech et al. [18–20], using the multivariable nonlinear least-squares computer fitting subroutine ZXSSQ in the IMSL statistical package (International Mathematical and Statistical Libraries, Houston, TX). In all cases, the variation of initial parameters was observed to have negligible effect upon the final solution. As a check to the validity of the fit, calculated curves based on the output parameters were displayed graphically along with the experimental points on the high-resolution graphics screen of an IBM Personal Computer. This procedure also allowed for real time interactive manipulation of parameters and was used to generate some of the calculated curves shown in the text.

All calculations involving multivariate fits were carried out on an IBM 4341 at the University of Texas Regional Computer Center.

3. Results and discussion

3.1. Kinetic analysis

A number of kinetic models have been previously formulated in order to account for the pH dependence of exchange rates measured from NMR experiments on amine systems capable of both proton exchange and amine inversion [15–17]. Of these, the model recently proposed by Delpuech et al. [18–20] appears to be capable of explaining experimental results over a wide variation in pH range, in both protic and aprotic solvents. The kinetic expressions presented in this section are special adaptations of the more general theory presented by these workers.

We shall consider the general appearance of NMR spectra of an amine in aqueous medium to display resonances arising from RN, RNH^+, RN* and RN^*H^+. Here RN and RN* represent the two diastereotopic methyl groups related by nitrogen inversion and RNH^+ and RN^*H^+, the protonated forms of these same amines. Since our experiments were performed well below the pK_a of the amine, it is not unreasonable to assume that resonances arising from the deprotonated forms will be absent. We may, thus, confine the kinetic treatment to the appearance of resonances arising only from RNH^+ and RN^*H^+.

In the absence of proton exchange (and nitrogen inversion), both RNH^+ and RN^*H^+ would be expected to give rise to separate well-resolved resonances. As the pH of the solution is raised, two separate exchange processes change the appearance of the observed spectrum. First, proton transfer between the protonated amine and other groups in solution results in exchange broadening of the resolved methyl resonances (RNH^+ and RN^*H^+). Second, the RNH^+ and RN^*H^+ resonances will continue to broaden with increasing pH until nitrogen inversion begins to interchange the two methyl positions resulting in coalescence of their resonances [24,25]. The exchange process may be written as

$$\mathrm{RNH^+} \overset{k_{\mathrm{NA}}}{\underset{k_{\mathrm{NB}}}{\rightleftharpoons}} \mathrm{RN^*H^+}$$

with k_{NA} and k_{NB} the respective kinetic rate constants, each of which is a function of kinetic rate constants for proton exchange and nitrogen inversion. One can assume that proton exchange proceeds through a generalized deprotonation-reprotonation scheme:

$$\mathrm{RNH^+} \xrightarrow{k_d} \mathrm{RN + H^+} \text{ and } \mathrm{RN + H'^+} \xrightarrow{k_p} \mathrm{RNH'^+}$$

and similarly,

$$\mathrm{RN^*H^+} \xrightarrow{k'_d} \mathrm{RN^* + H^+} \text{ and } \mathrm{RN^* + H'^+} \xrightarrow{k'_p} \mathrm{RN^*H'^+}$$

with the inversion of the nonprotonated amine given by:

$$\mathrm{RN} \overset{k_{\mathrm{A}}}{\underset{k_{\mathrm{B}}}{\rightleftharpoons}} \mathrm{RN^*}$$

By applying steady-state rate equations to the three-step kinetic scheme involving conversion from $\mathrm{RNH^+}$ to $\mathrm{RN^*H^+}$ and vice versa, it can be shown [20] that

$$k_{\mathrm{NA}} = k_d k_{\mathrm{A}} k'_p / D \qquad (1\mathrm{A})$$

$$k_{\mathrm{NB}} = k'_d k_{\mathrm{B}} k_p / D \qquad (1\mathrm{B})$$

where $D = k_p k_{\mathrm{B}} + k_p k'_p + k_p k_{\mathrm{A}}$.

We have considered the following specific mechanisms for deprotonation of the tertiary amines of tetramethyllysine and its methyl ester in aqueous solvent:

$$\mathrm{RNH^+ + OH^-} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \mathrm{H_2O + RN} \qquad (2\mathrm{A})$$

$$\mathrm{RNH^+ + H_2O} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \mathrm{H_3O^+ + RN} \qquad (2\mathrm{B})$$

$$\mathrm{RNH^+ + RN} \overset{k_3}{\underset{k_3}{\rightleftharpoons}} \mathrm{RN + RNH^+} \qquad (2\mathrm{C})$$

$$\mathrm{RNH^+ + RCO_2^-} \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} \mathrm{RN + RCO_2H} \qquad (2\mathrm{D})$$

where eq. 2C represents intermolecular proton transfer between α-amines and eq. 2D proton transfer (inter- or intramolecular) which occurs only for the free acid ($k_4 = k_{-4} = 0$ for TMLME). Loewenstein and Meiboom [26] have shown that proton transfers between amines (as depicted in eq. 2C) occur through in interstitial water molecule. One might similarly imagine water to mediate the transfer from a protonated amine to a free carboxylate anion (eq. 2D). However, since we have not carried out the proper ^{1}H-NMR experiments to confirm such a mechanism for lysine derivatives in aqueous solvent, we have simply assumed the direct transfer mechanism. If these proton transfers do, in fact, involve an interstitial solvent molecule, then the rate constants k_3, k_4 and k_{-4} must be rewritten in terms of rates of proton transfer to and from the solvent.

Considering the above deprotonation schemes, we find

$$k_d = k_1[\mathrm{OH^-}] + k_2[\mathrm{H_2O}] + k_3[\mathrm{RN}] + k_4[\mathrm{RCO_2^-}] \qquad (3\mathrm{A})$$

$$k_p = k_{-1}[\mathrm{H_2O}] + k_{-2}[\mathrm{H^+}] + k_3[\mathrm{RNH^+}] + k_{-4}[\mathrm{RCO_2H}] \qquad (3\mathrm{B})$$

Since most work was carried out one to two pH units below the pK_a of the titrating tertiary amine group of interest, we shall assume $[\mathrm{RNH^+}] = C_0$, the total concentration of the lysine derivative in solution. Substituting for K_w, the ionization constant of water and for K_{a1} and K_{a2}, the acid dissociation constants for the carboxylate and amine groups, respectively,

$$k_d = \frac{k_1 K_w}{[\mathrm{H^+}]} + k_2[\mathrm{H_2O}] + \frac{k_3 K_{a2} C_0}{[\mathrm{H^+}]} + \frac{k_4 C_0 K_{a1}}{(K_{a1} + [\mathrm{H^+}])} \qquad (4\mathrm{A})$$

$$k_p = k_{-1}[\mathrm{H_2O}] + k_{-2}[\mathrm{H^+}] + k_3 C_0 + \frac{k_{-4} C_0 [\mathrm{H^+}]}{(K_{a1} + [\mathrm{H^+}])} \qquad (4\mathrm{B})$$

At equilibrium, the second-order rate constants k_{-1}, k_{-2} and k_{-4} must be subject to the conditions of microscopic reversibility, so that

$$\frac{k_1}{k_{-1}} = \frac{[\mathrm{RN}][\mathrm{H_2O}]}{[\mathrm{RNH^+}][\mathrm{OH^-}]} = \frac{K_{a2}}{K_w}[\mathrm{H_2O}] \qquad (5\mathrm{A})$$

$$\frac{k_2}{k_{-2}} = \frac{[\mathrm{H_3O^+}][\mathrm{RN}]}{[\mathrm{RNH^+}][\mathrm{H_2O}]} = \frac{K_{a2}}{[\mathrm{H_2O}]} \qquad (5\mathrm{B})$$

$$\frac{k_4}{k_{-4}} = \frac{[\mathrm{RN}][\mathrm{RCO_2H}]}{[\mathrm{RNH^+}][\mathrm{RCO_2^-}]} = \frac{K_{a2}}{K_{a1}} \qquad (5\mathrm{C})$$

If there are equal populations of RNH^+ and RN^*H^+ in solution, then $k_p = k'_p$, $k_d = k'_d$ and $k_A = k_B = k_i$ in eq. 1. This leads to the result that the overall rate of exchange between the two populations, as measured by NMR, is given by

$$k_N = \frac{2k_d k_i}{2k_i + k_p} \tag{6}$$

3.2. Experimental data for model compounds

Fig. 1 shows the proton-decoupled ^{13}C-NMR spectra of TML in water. The two resonances at 43.1 and 41.9 ppm in the spectrum at pH 10 (fig. 1A) can be assigned to methyl groups on the ϵ- and α-nitrogens, respectively, on the basis of chemical shifts taken from the literature for N^ϵ,N^ϵ-dimethyllysines and N^α,N^α-dimethylamines in various peptides and proteins [1–5]. As the pH is lowered, the α-dimethylamine resonance (at 41.9 ppm, pH 10) splits into separate, well resolved resonances of equal intensity with limiting chemical shifts of 40.4 and 43.5 ppm at pH 2. These can be assigned to the methyl groups on each of the two diastereotopic positions on the α-amine related to one another by nitrogen inversion. The ϵ-dimethylamine resonance also splits into two peaks as the pH is lowered but the chemical shifts of the two diastereotopic ϵ-methyl groups are too similar to allow complete resolution of these two resonances at pH 2.

Fig. 2 shows the observed chemical shifts of the diastereotopic N^α- and N^ϵ-methyl carbons of TML and TMLME as a function of pH. These shifts indirectly reflect the pH at which the diastereotopic carbons pass from the fast-exchange regime on the NMR time scale into the slow-exchange regime. Within the limits of resolution attainable, the pH-dependent behavior of the N^ϵ-methyl carbon resonances of TML is identical to that of N^ϵ-methyl resonances in TMLME. In contrast, the pH-dependent chemical shift behavior of the N^α-methyl resonances of TML is strikingly different from that of N^α-methyl resonances of TMLME. Hence, although the experimentally determined pK_a for the α-amino group of TMLME is only one unit below the corresponding pK_a for TML (see below) the pH value at which the diastereotopic N^α-methyl carbons of TMLME pass from the fast- to the slow-exchange regime lies about 4.5 pH units below the corresponding exchange transition of TML.

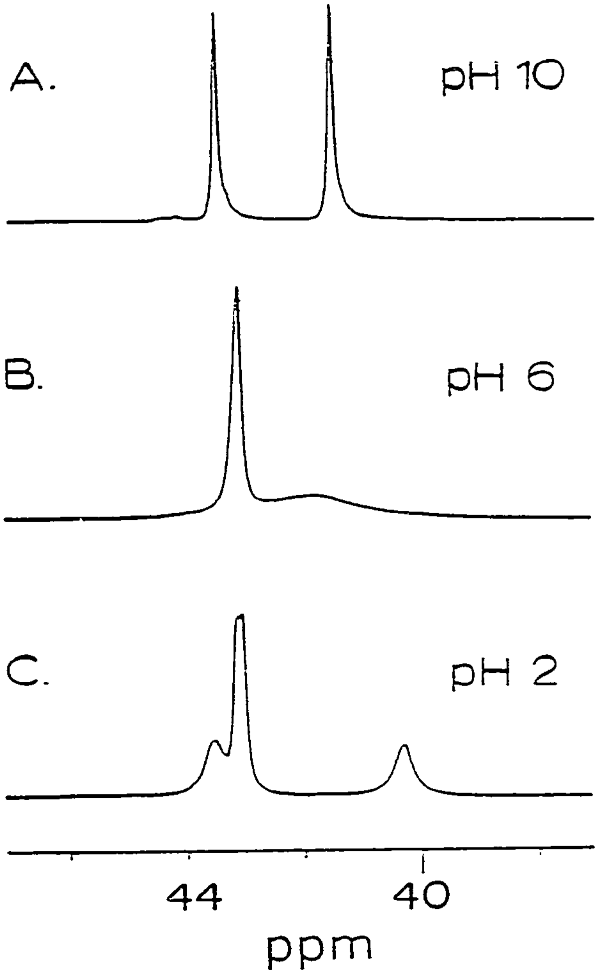

Fig. 1. Proton-decoupled ^{13}C-NMR spectra of 60 mM TML in H_2O under pH conditions shown in each portion of the figure.

Using computer simulation techniques, the line widths of the ^{13}C-methyl resonances of our model compounds could be analyzed to yield lifetimes for the exchange of the diastereotopic methyl groups (ref. 24; and use of DNMR3IT$_2$ program).

This was most easily accomplished for the α-amino substituted methyl resonances, where separate resonances for each of the two methyl populations were fully resolved. A high pH limit was attained for those spectra, yielding reliable rate data when the line width contribution due to exchange was less than the contribution to the line width arising from transverse relaxation and magnetic field inhomogeneity (about 2 Hz). The logarithms of the exchange rates obtained from line width analysis of the α-dimethylamine resonances in spectra of TML and TMLME ester are shown as calculated points as a function of pH in fig. 3.

Lines drawn closest through the points in fig. 3 represent best fits to the data calculated by simultaneous variation of microscopic rate constants k_2, k_3, k_4 and k_i. Because calculated fits were insensitive to variations in k_1 up to 10^{12} M^{-1} s^{-1}, a value of 10^9 M^{-1} has been assumed in all calculations. This value is less than the upper limit for k_1 of

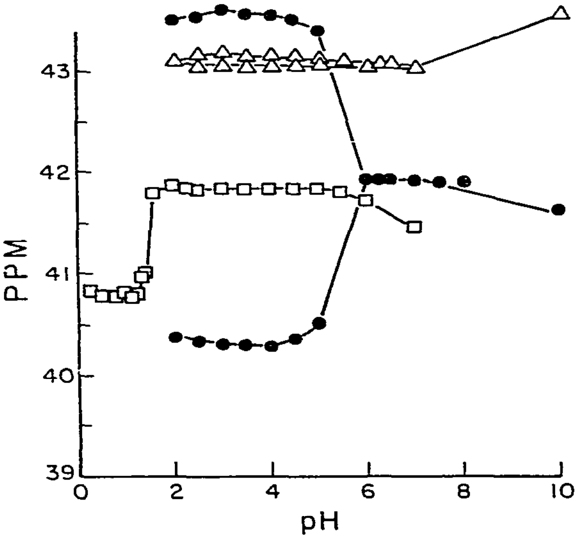

Fig. 2. The pH dependence of the ^{13}C chemical shifts of resonances arising from the N^{α}-amine methyl groups of TML (●) and TMLME (□) and the N^{ϵ}-amine methyl groups of TML and TMLME (△). Lines drawn between experimental points were extrapolated from experimental data. The concentration of both compounds was 60 mM.

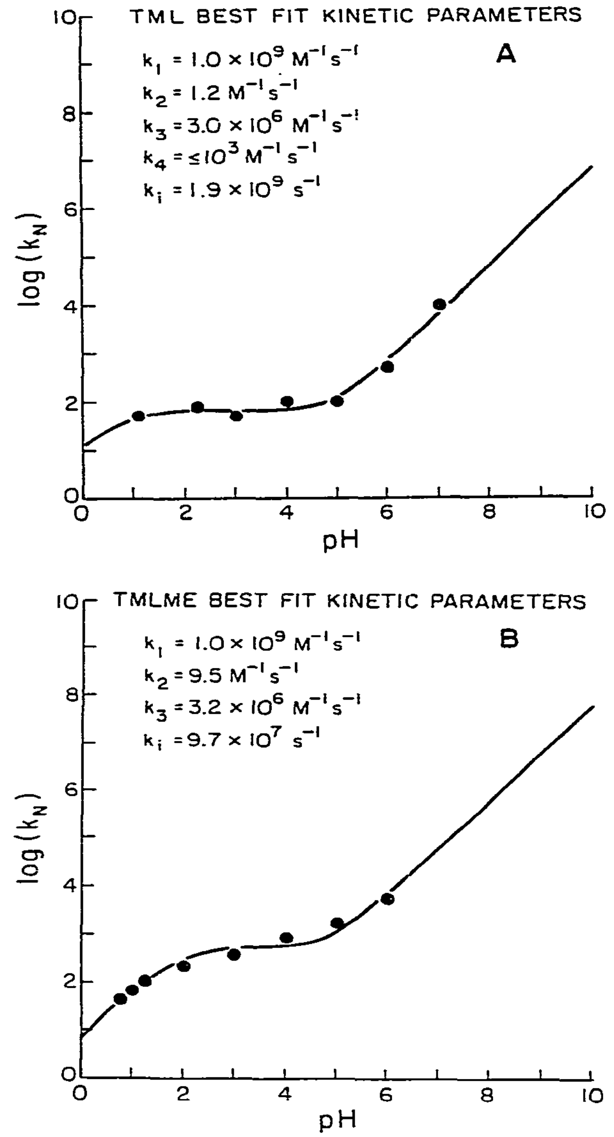

Fig. 3. pH dependence of rate data (shown as points) obtained by fitting line widths of the α-dimethylamine resonances in ^{13}C-NMR spectra of (A) TML and (B) TMLME. Curves drawn through experimental points represent best fits to the kinetic data. Forward rate constants obtained from the fit are shown in each of the figures.

10^{10} M^{-1} s^{-1} as determined by Loewenstein and Meiboom [26]. Reverse rate constants, k_{-1}, k_{-2} and k_{-4}, were related to forward rate constants, k_1, k_2 and k_4 by means of relationships involving appropriate acid dissociation constants (eqs. 5A–C). pK_a values for the α-amino groups of the lysine derivative and its methyl ester were determined by potentiometric titration to be 8.5 and 7.5, respectively. The pK_a of the carboxyl group of TML was assumed identical to the pK_a of the underivatized amino acid ($pK_a = 2.18$) [27].

In general, each of the best-fit curves in fig. 3 have two linear regions, one below pH 2 and the other above pH 4, and a plateau region, extending from pH 2.5 to 4. Consider first the region below pH 2, where $k_p > k_i$. Referring to eqs. 4A and 4B, it may be seen that in this region k_p is limited by $k_{-2}[H^+]$ while k_d appears to be limited by $k_2[H_2O]$. Combining these two conditions along with eq. 6 leads to the limiting law first proposed by Saunders and Yamada [15]. $k_N = 1/\tau = 2k_iK_{a2}/[H^+]$. Extrapolation of a log-log plot of this equation to obtain a y-intercept yields a value for k_i, given a known acid dissociation constant. In the pH region above pH 2, $2k_i > k_p$ and $k_N = k_d$. The proton dissociation rate constant, k_d, is limited by the rate term $k_2[H_2O]$ below pH 4 and by the rate term $k_3K_{a2}C_0/[H^+]$ in the limit of high pH. Such behavior gives rise to the plateau region between pH 2 and 4 and the linear region above pH 4. The y-intercept obtained from extrapolation of the plateau region and the linear region above pH 4 should yield values for k_2 and k_3, respectively.

Despite similarities in the general pH-dependent behavior of $\log(k_N)$ for TML and TMLME, there are marked differences. For example, fig. 3A shows that $\log(k_N)$ for the α-dimethylamine resonance of TML decreased from a value near 4.0 at pH 7 to a value near 2.0 at pH 5, thereafter remaining essentially unchanged down to pH 1. On the other hand, analogous $\log(k_N)$ values determined for the methyl ester of this compound decrease rather slowly from an upper limit of 3.75 at pH 6 into a pseudoplateau region between pH 5 and 2, thereafter decreasing quite readily as a function of pH below pH 1.5.

Rate constants determined by fitting the pH dependence of k_N for either of the two model compounds are listed at the upper left of the plots in fig. 3. Considering the markedly different types of pH-dependent behavior of $\log(k_N)$ observed when comparing the data for TML to those of TMLME, the agreement in rate constants determined from fits to either of the two data sets is really quite good. For example, k_2 determined from fits of the rate data for TML is 1.2 M^{-1} s^{-1} compared to 9.5 M^{-1} s^{-1} determined from analogous rate data for TMLME. The rate constant for proton exchange between amine groups, k_3, is predicted to be about 3×10^6 M^{-1} s^{-1} for both model compounds. The rate constant showing the greatest disparity between the two compounds is k_i, the amine inversion rate constant.

Rate constants determined from the fitting routine based on the kinetic model used in this study are also in reasonable agreement with those determined by other workers for tertiary amines in water. Values previously obtained for k_2, the proton transfer rate constant to water, range from 0.06 M^{-1} s^{-1} for trimethylammonium [26] to 500 M^{-1} s^{-1} for *N*-benzyl-*N*-methylethanolamine [16]. The latter value has been reinterpreted by Delpuech and Bianchin [21] to be of the order of 50 M^{-1} s^{-1}, based on their steady-state kinetic model. In any case, our value for k_2 of about 1–10 M^{-1} s^{-1} falls well within this range. Rate constants previously determined for proton transfer between amines in water range from about 6×10^6 M^{-1} s^{-1} for *N*,*N*′-dimethylpiperazine to about 6×10^8 M^{-1} s^{-1} for trimethylammonium [16,17,21]. Admittedly, our value for k_3 (3×10^6 M^{-1} s^{-1}) lies on the lower limit of this range. It is quite likely that this disparity could result from the difficulty inherent in fitting line widths in our spectra at high pH, where the exchange contribution to the line width is relatively small. Values obtained from the rate data of both model compounds for k_i are in reasonable agreement with nitrogen inversion rates predicted theoretically [11] and determined experimentally [15–18].

Most noteworthy regarding the entire kinetic analysis of rate data for the two model compounds is that it appears to be possible to explain the different pH dependence of $\log(k_N)$ almost entirely by the difference in amine acid dissociation constants. Hence, additional mechanisms such as the proton transfer to a carboxylate group (eq. 2D) need not be included in the case of TML in order to explain the pH-dependent rate data. In fact, introducing k_4 in place of k_2 as a limit to k_d results in a fit to experimental data having a least-squares residual 100-times that of the best fit. Using our value for k_2 of 1.2 $M^{-1}\ s^{-1}$ and the condition that $k_4 > k_2[H_2O]/C_0$ to affect significantly the appearance of the calculated results, we estimated that k_4 would need to be greater than $10^3\ M^{-1}\ s^{-1}$.

3.3. Experimental data for reductively methylated Con A

Fig. 4 shows the proton-decoupled ^{13}C-NMR of native Con A following reductive methylation with [^{13}C]formaldehyde and $NaCNBH_3$. Under the conditions used in the reaction, reductive methylation with $NaCNBH_3$ has been shown to be specific for N-terminal α-amino and lysyl ε-amino groups [1]. On the basis of their pH titration behavior in the native and denatured forms of the protein, the envelope of resonances at 43.1 ppm and the single resonance at 41.8 ppm may be assigned, respectively, to 11 of the 12 dimethyllysine residues and the single N-terminal dimethylalanine residue in Con A [6]. The two additional resonances observed at 40.9 and 44.1 ppm in the spectra of the protein at pH 5.6 have tentatively been assigned to the two ε-methyl groups of Lys-101. Magnetic nonequivalence of these two resonances is thought to result from the formation of a salt bridge between Lys-101 and Asp-203, so as to place the two methyl groups in significantly different magnetic environments. With decreasing pH, these two resonances sharpen and titrate into the envelope of dimethyllysine resonances with a single pK_a near to that expected of a titrating carboxyl side chain (pK_a = 3.25). This behavior is characteristic of that expected for increasingly rapid exchange between magnetically nonequivalent sites [29] brought about by protonation of the carboxylate side chain and hence, a gradual weakening of the proposed salt bridge.

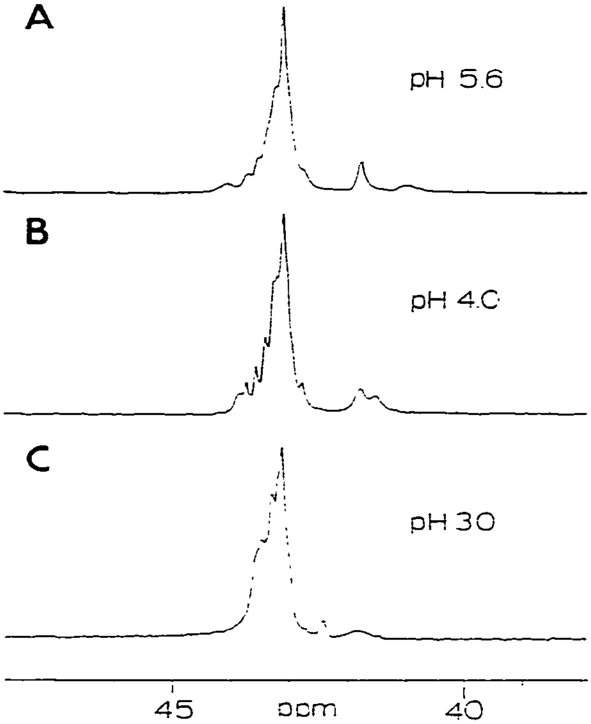

Fig. 4. Proton-decoupled ^{13}C-NMR spectra of reductively methylated Con A at several pH values. The total protein monomer concentration was 0.8 mM.

Changes in pH are also seen to affect the line width of the N-terminal dimethylamine resonance at 41.8 ppm and the resolution of dimethyllysine resonances comprising the envelope at 43.1 ppm. The enhanced resolution observable in the dimethyllysine envelope at pH 4 is probably a result of shifts in some of the dimethyllysyl resonances due to the titration of side chain carboxyl groups lying near to lysine side chains in the protein structure. The resolution is lost at pH 3, owing to the magnetic equivalence of many of these titrating groups, and again at pH 5.6, as a result of line broadening. Above pH 5.6, these resonances again sharpen so as to make possible their assignment to specific lysyl groups in the protein sequence [6]. The line-broadening effects observed for these resonances at intermediate pH values are most likely due to proton exchange between the lysyl ε-amino groups and other nearby titrating side chains in the protein.

As the pH is lowered from 5.6 to 3.0, the N-terminal dimethylalanine resonance is observed to broaden in a manner similar to the broadening observed for corresponding resonances in the spectra of the model compounds. Line widths of this resonance measured at half-height ($\Delta W_{1/2}$) and corresponding pH values are presented in columns 1 and 2 of table 1. At pH 5.0 and above, contributions to the line width are seen to arise only from instrumental inhomogeneity and transverse relaxation. Below pH 5.0, additional exchange contributions broaden the resonance.

The lifetimes of each of the protonated diastereotopic methyl populations (τ_A and τ_B) can be estimated from the observed line widths of the N-terminal dimethylamine resonance using the relationship valid in the near-fast-exchange regime [29]:

$$\pi\Delta W_{1/2} = \frac{N_A}{T_{2A}} + \frac{N_B}{T_{2B}} + N_A^2 N_B^2 (\omega_A^0 - \omega_B^0)^2 (\tau_A + \tau_B)$$

where N_A and N_B represent the mole fractions of each diastereotopic methyl population and ω_A^0, ω_B^0, T_{2A} and T_{2B}, the angular frequencies and transverse relaxation times of the resonances arising from each of the two populations in the absence of exchange, respectively. Results of the calculation are tabulated in column 3 of table 1, assuming $1/\tau_A = 1/\tau_B = k_N$, $N_A = N_B = 0.5$, $1/\pi T_{2A} = 1/\pi T_{2B} = 6.3$ Hz and $(\omega_A^0 - \omega_B^0)$ given by the shift difference taken from our studies on TMLME ($\nu_A^0 - \nu_B^0 = 110$ Hz). A plot of $\log(k_N)$ vs. pH was seen to be linear with unit slope above pH 4.0, a condition fulfilled for base-catalyzed nitrogen inversion either in the limit of high pH where $k_N = k_d$, or in the limit of low pH where $k_N = 2k_i k_a/[H^+]$. The $\log(k_N)$ value at pH 3 was seen to lie 0.35 log units above the extrapolated linear portion of the plot, inferring a transition into a plateau region and supporting the former of the two limiting conditions.

Table 1

Line widths and calculated rate constants determined from the N-terminal ^{13}C-methyl amine resonance of reductively methylated Con A at various pH values

$\Delta W_{1/2}$ is taken from the line width measured digitally at half-height and has an error of ±0.5 Hz. k_N was calculated from $\Delta W_{1/2}$ assuming near-fast exchange. $k_t^{CO_2^-}$ is the rate constant associated with proton transfer from the N-terminal amine to two carboxylate side chains with assumed pK_a values of 4.7.

pH	$\Delta W_{1/2}$ (Hz)	$\log(k_N)$	$k_t^{CO_2^-}$ ($\times 10^{-4}$) (s^{-1})
3.0	22.5	3.08	3.0
4.0	11.7	3.59	1.2
4.5	8.8	3.98	1.2
5.0	6.8	–	–
5.5	6.8	–	–

A number of possibilities exist for the acceptor of the proton transferred from the N-terminal amino group. On the basis of proton transfer mechanisms dominant in the model compounds between pH 2 and 4, it seems reasonable that the proton from the N-terminal amine of the protein is transferred to a surrounding solvent molecule. Assuming that such a transfer is much faster than transfer to other nearby groups in the protein yields a value for $k_t^{H_2O}$, where $k_d = k_N = k_t^{H_2O}[H_2O]$. Such an assumption would be valid only below pH 4.0, where a plot of $\log(k_N)$ vs. pH falls into a plateau region (see fig. 3). Taking the value of k_N obtained from the data at pH 3 yields a value for $k_t^{H_2O}$ of about 20 M^{-1} s^{-1}, a value which compares quite favorably with k_2 determined for the model compounds (1–10 M^{-1} s^{-1}). The pH dependence of the observed exchange rate above pH 4.0 infers that these rates must be limited by proton transfer to other nearby ionizable groups. Reference to the three-dimensional crystal structure of native Con A shows that Asp-2 and Asp-218 have their carboxyl side chains positioned 5.5 and 5.8 Å, respectively, from the N-terminal, making them likely proton acceptors below their intrinsic pK_a values [30]. Inferring from titration data of carboxyl side chains in other proteins [31], the pK_a values of these groups can be estimated to be about 4.7, roughly the same pH at which broadening of the N-terminal dimethylamine resonance becomes evident in our spectra of Con A. Assuming proton transfer to these nearby aspartic acid side chains, an estimate of the proton transfer rate constant, $k_t^{CO_2^-}$ is possible using the limiting expression, $k_N = k_d = 2k_t^{CO_2^-}K_a/(K_a + [H^+])$. Results of the calculation are presented in column 4 of table 1 for pH values below 4.7, the intrinsic pK_a assumed for both aspartic acid side chains.

Because of the problems inherent in obtaining rate data from spectra of Con A acquired below pH 3, estimations of rate constants for the deprotonation of N-terminal amine by the solvent (k_2) and for amine inversion (k_i) are, at best, of limited value. However, if one assumes that the value of k_N obtained from the data at pH 3 is indeed limited by $k_2^{H_2O}[H_2O]$, the point at which k_p becomes determined by k_i must lie below pH 3. This implies a lower limit for k_i of about 10^7 s^{-1}.

4. Concluding remarks

The pH dependence of the line broadening of α-dimethylamine resonances in ^{13}C-NMR spectra of TML and TMLME can be satisfactorily explained assuming a steady-state kinetic model for base-catalyzed nitrogen inversion. Differences between these two model compounds in terms of their pH-dependent exchange behavior can be adequately explained by differences in acid dissociation constants of their respective α-amino groups. Under limiting conditions of high or low pH, the observed exchange behavior was used to determine rate constants for proton transfer to a corresponding unprotonated amine (k_3) or the rate constant for amine inversion (k_i). At intermediate pH values the exchange between methyl populations was found to be limited by amine deprotonation by the solvent (k_2). From our studies on TML, it appears that deprotonation of the α-amino group by transfer to the titrating carboxyl group is a relatively unimportant proton-exchange mechanism. An upper limit for the rate constant associated with this mechanism was estimated to be about 10^3 M^{-1} s^{-1}, compared to 10^6 M^{-1} s^{-1} for k_3 and 1–10 M^{-1} s^{-1} for k_2. The same kinetic analysis could not be done on the ϵ-dimethylamine data because the methyl groups on the ϵ-amine do not have significantly different chemical shifts. However, these resonances appeared, qualitatively, to show pH-dependent broadening effects similar to those observed for the α-dimethylamine resonances.

The pH-dependence of the resolved N-terminal dimethylalanyl resonance in methylated Con A was analyzed in terms of the same kinetic model which was used to account for broadening of the N^{α}-amine methyl resonances in the model compounds. Below pH 4.0, broadening of this resonance could be explained in terms of base-catalyzed nitrogen inversion, where the proton of the N-terminal methyl amine was transferred to the solvent. Above pH 4.0, the data are consistent with a kinetic model in which proton transfer occurs to two nearby carboxylate groups in the protein (Asp-2 and Asp-218). There is no obvious reason as to why this transfer mechanism might be dominant in the protein and relatively insignificant for the model compound, TML. It is possible, however, that the carboxylate side chains are so positioned in the protein as to make proton transfer through an interstitial water molecule an effective amine deprotonation mechanism.

A final comment is in order regarding a ^{15}N spin-lattice relaxation contribution arising from exchange between two diastereotopic populations via a protonation-reprotonation scheme. We have estimated nitrogen inversion rates to be approx. 10^8–10^9 s^{-1} for the α-amine in our model compounds and greater than 10^7 s^{-1} for the N-terminal amino group of Con A. These first-order rates are of the same order of magnitude as the N^{15} Larmour frequency at commonly used magnetic field strengths, and would be expected to contribute to the ^{15}N spin-lattice relaxation time (T_1). Such an exchange contribution to the ^{15}N T_1 values has recently been verified for the ^{15}N-terminal amine of a tetrapeptide [32]. Studies are presently underway in order to assess the exchange contribution to the T_1 values of ^{13}C-methyl resonances of reductively methylated Con A.

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