Hydrogen Bond Stabilities in the Isolated Alamethicin Helix: pH-Dependent Amide Exchange Measurements in Methanol

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Abstract: To determine the stability of individual hydrogen bonds in the helical ion channel peptide alamethicin in methanol, pH-dependent hydrogen−deuterium exchange rates were measured using nuclear magnetic resonance (NMR) spectroscopy. pH-dependent exchange data were also measured for the protected dipeptides *N*-acetyl-K-X-NH_2, where X is G, A, or Aib (α-aminoisobutyric acid) and *N*-acetyl-A-A-NH_2 to characterize exchange parameters for non-hydrogen-bonded amides in methanol. The pH-dependent amide exchange data for alamethicin and the dipeptides were fit to an equation defining acid and base catalysis by solvent. Electrostatic effects arising from the peptide helix macrodipole are shown to affect (by up to 20-fold) the acid- and base-catalyzed exchange rate constants for amides at the helix termini. Amide exchange protection factors for the alamethicin amides were determined either from the ratio of $k_{min}/k_{min(free)}$ (where k_{min} and $k_{min(free)}$ are the exchange rate constants at the minimum of the pH-dependent plots for alamethicin amides, and a non-hydrogen-bonded amide, respectively) or by correcting acid- and base-catalyzed protection factors for sequence-dependent inductive and steric effects. The enhancement of acid-catalyzed, relative to base-catalyzed, exchange protection factors supports the imidic acid mechanism for acid-catalyzed exchange (Perrin, C. L. *Acc. Chem Res.* **1989**, *22*, 268−275) for alamethicin in methanol. The exchange protection factors are a measure of the stability of each of the hydrogen bonds of alamethicin with respect to exchange-limiting backbone fluctuations, and indicate that the peptide has stable hydrogen bonds throughout the helical structure. The high exchange stability of Aib3 and V15 NH's indicates that these amides are protected by 3_{10} helical hydrogen bonds. In contrast to melittin (Dempsey, C. E. *Biochemistry* **1992**, *31*, 4705−4712) the P14 residue of alamethicin does not markedly disrupt hydrogen bonding around the center of the alamethicin helix. The exchange data demonstrate that an internal proline residue may have quite different effects on the structure and dynamics of an α-helix depending on the peptide sequence around it.

Introduction

The analysis of the kinetics of exchange of peptide amide hydrogens with solvent is a powerful method for determining structural and dynamic properties of polypeptides in solution. In combination with high-resolution NMR[1] spectroscopy the stabilities of individual hydrogen bonds may be determined as well as information on backbone dynamics, since exchange of hydrogen-bonded amides requires hydrogen bond openings that occur during fluctuations of the polypeptide backbone.[2] Analysis of pH-dependent amide exchange from helical polypeptides has been used to define the effects of an internal proline residue on hydrogen bond stabilities in the ion channel peptide melittin in methanol[3] and to analyze the helix−coil transition and the fraying of peptide bonds at the N- and C-termini of a simple alanine-based peptide[4] (see also ref 5). The hydrogen bond stabilities determined in these studies seem to reflect intrinsic properties of the respective polypeptide backbones since good correlations with theoretical calculations were found for the alanine-based polypeptide in water[4] and with hydrogen bond stabilities determined in molecular dynamics simulations for melittin.[6]

A major goal in hydrogen exchange analysis is the determination of an exchange protection factor (PF) in which the effects of hydrogen bonding are isolated from all other influences on amide exchange. In the general scheme (eq 1)[2a] in which

$$\mathrm{NH(c)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{NH(o)} \xrightarrow{k_3} \mathrm{ND(o)} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \mathrm{ND(c)} \qquad (1)$$

hydrogen−deuterium exchange is analyzed in terms of transient fluctuations between hydrogen-bonded "closed" conformers (in which exchange is completely suppressed) and "open" conformers (in which the amide exchanges with characteristics of a free amide), the exchange protection factor is then equivalent to the equilibrium constant ($K_{op} = k_1/k_2$) that characterizes the exchange-limiting backbone fluctuation (*i.e.*, $PF = 1/K_{op}$). The equilibrium constant is usually the only accessible parameter (rather than k_1 and k_2) in exchange measurements because the fluctuation leading to exchange is, in most cases, in preequilibrium with the chemical exchange step; that is, $k_2 \gg k_3$.[2a,c]

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(1) Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; k_a, acid-catalyzed exchange rate constant; k_b, base-catalyzed exchange rate constant; k_{min}, exchange rate constant at the minimum of the pH-dependent exchange rate curve; $k_{min(free)}$, k_{min} for an amide in an unstructured polypeptide, taken here to be *N*-Ac-A-A-NH_2; NMR, nuclear magnetic resonance; PDLA, poly-D,L-alanine; PF, exchange protection factor; pH*, uncorrected pH measured with the hydrogen electrode calibrated with aqueous pH buffers; pH_{min}, pH at which the experimental exchange rate constant is a minimum; Phol, phenylalaninol; r_{min}, ratio of $k_{min(free)}/k_{min(c)}$, where $k_{min(c)}$ is k_{min} for an amide corrected for sequence-dependent steric effects as in Table 4; U, one letter code for α-aminoisobutyric acid.[11c]

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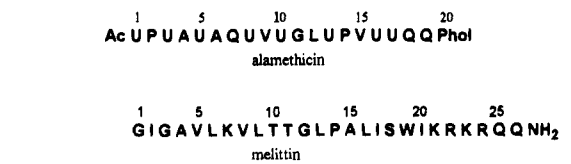

Figure 1. Amino acid sequences of melittin and the alamethicin fraction used in this study. The letter U is used for α-aminoisobutyric acid (Aib).[11c] Variants of alamethicin have U replacing A6 or E replacing Q18.

Exchange protection factors are normally measured as the suppression of the base-catalyzed exchange rate constant (k_b) relative to k_b for a model unstructured peptide in which the amide is not hydrogen bonded.[2c] Corrections are made for sequence-dependent inductive[7,8] and steric[8] effects that alter the stability of the base- (and acid-) catalyzed exchange intermediates and thus affect k_b (and k_a) for amides independent of hydrogen bonding. In conditions where exchange data from model peptides are less well characterized, protection factors may be determined by averaging uncorrected protection factors based on suppression of acid- and base-catalyzed exchange rate constants.[3] This method relies on the observation that inductive[8] and electrostatic effects[9] have roughly equal and opposite effects on the acid- and base-catalyzed exchange rate constants so that the former effects are approximately canceled. The latter method is beneficial in potentially eliminating effects of inductive and electrostatic contributions to exchange but is susceptible to errors resulting from the contribution of imidic acid chemistry[10] in the acid-catalyzed exchange mechanism.

In a study of the effects of internal proline residues on the conformational and dynamic properties of ion channel peptides, we have measured extensive pH-dependent amide exchange rates for alamethicin in methanol. Alamethicin, from *Trichoderma viride*, is a 20 amino acid peptide, rich in α-aminoisobutyric acid (Aib) (Figure 1), which diffuses into membranes and induces voltage-dependent ion conductance.[11] The proline-14 residue is expected to cause a disruption of helical structure in this peptide, although studies of the monomeric peptide in methanol have been equivocal about the extent of this disruption.[12] The question is of interest because some models of voltage activation of alamethicin, from inactive ("prepore") states to conducting pores, involve recruitment of disordered structure around, and C-terminal to, the P14 residue into a helix as a voltage sensor.[13]

In this paper we consider the optimal method for measuring estimates of K_{op} from amide exchange data in methanol and characterize some of the conformation-dependent electrostatic contributions to exchange from helical peptides. The data lead to a description of the hydrogen bond stabilities of the alamethicin helix in a solvent where intramolecular hydrogen bonding is favored, which indicates that the P14 residue has quite different effects on the structure and dynamics of the melittin (Figure 1) and alamethicin helices.

Experimental Section

Materials. An extract of alamethicin from *T. viride* was purchased from Sigma. Deuteriomethanol (CD_3OD and CD_3OH), sodium acetate-d_3, and D_2O were from Aldrich Chemical Co. All other chemicals were of research grade or higher. The N-terminally-acetylated and C-terminally-amidated dipeptides *N*-Ac-K-G-NH_2, *N*-Ac-K-A-NH_2, and *N*-Ac-K-U-NH_2 were synthesized by Dr. G. Bloomberg of the Bristol University Centre for Molecular Recognition. *N*-Ac-K-[^{15}N]A-NH_2 and *N*-Ac-[^{15}N]A-A-NH_2 were synthesized by Dr. Bloomberg with [^{15}N]-alanine prepared in the laboratory of Dr. C. Willis (Bristol University Chemistry Department and Molecular Recognition Centre). The protected dipeptides were desalted by gel filtration on Sephadex G-15 (Pharmacia, U.K.) in 2% aqueous acetic acid before NMR measurements.

Methods. Purification of Alamethicin. The two major components of the commercial extract are alamethicin having the amino acid sequence given in Figure 1 (alamethicin-A6, 35−40%) and alamethicin-U6 (30−35%). These components were isolated by reversed phase HPLC on a Vydac C4 semipreparative column using a linear gradient of 0.1% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) (Dempsey, C. E.; Handcock, L. J. Submitted for publication). The alamethicin-A6 component was purified to greater than 95% homogeneity as determined by NMR spectroscopy and was used for all the measurements described here.

Amide Exchange Measurements. Amide exchange measurements with alamethicin were made immediately after dissolving the peptide in 0.5 mL of CD_3OD at a concentration between 0.5 and 1.5 mM. Samples of the peptide were previously lyophilized from CH_3OH adjusted over a range of pH values to give a series of samples covering pH* −0.5 to +7.0 (where pH* is the apparent pH using a hydrogen electrode calibrated with aqueous buffers). Each sample was immediately put into a 5 mm NMR tube, and one-dimensional ^{1}H NMR spectra were obtained over an exchange time course at 20 °C using the Jeol Alpha 500 MHz NMR spectrometer of the Bristol University Molecular Recognition Centre. Depending on the pH*, amide exchange measurements were made over a period of minutes to up to several months. For long time series, samples were kept at 20 °C in a thermostated water bath between spectral accumulation. Two-dimensional NMR spectra to confirm spectral assignments were obtained using standard conditions.

Amide exchange measurements with the N- and C-terminally-protected dipeptides were made over a restricted range of pH* values accessible to measurement of time-resolved exchange rates. It was generally difficult to predict the pH* of solutions of these peptides in deuteriomethanol after lyophilizing from pH-adjusted water. Amide exchange with the dipeptides was therefore measured in CD_3OD buffered with 2 mM glycine which buffers between pH* 2.5 and pH* 4.5 in methanol. Exchange rates measured in deuteriomethanol with and without 2 mM glycine buffer were the same within experimental error. The amide signals of K1 and A2 in *N*-Ac-K-A-NH_2 are exactly coincident in methanol; peptide incorporating [^{15}N]A2 was therefore used for amide exchange measurements with this peptide to resolve the amide signals (Figure 2).

Amide hydrogen−deuterium exchange rates can be measured accurately (with an error of less than 5%) by time-resolved exchange measurements in methanol, and the greatest error in determining pH*-dependent exchange data in methanol is in the measurement of pH*. The use of the hydrogen electrode for measuring pH* in methanol has previously been discussed with reference to amide exchange analysis.[3a] After completion of an exchange series, the pH* was measured using the following protocol which gave reproducible pH measurements of deuteriomethanolic solutions. The pH electrode was calibrated with aqueous pH buffers, and the probe was rinsed and lightly dried with tissue. The probe was then immersed briefly in methanol and dried

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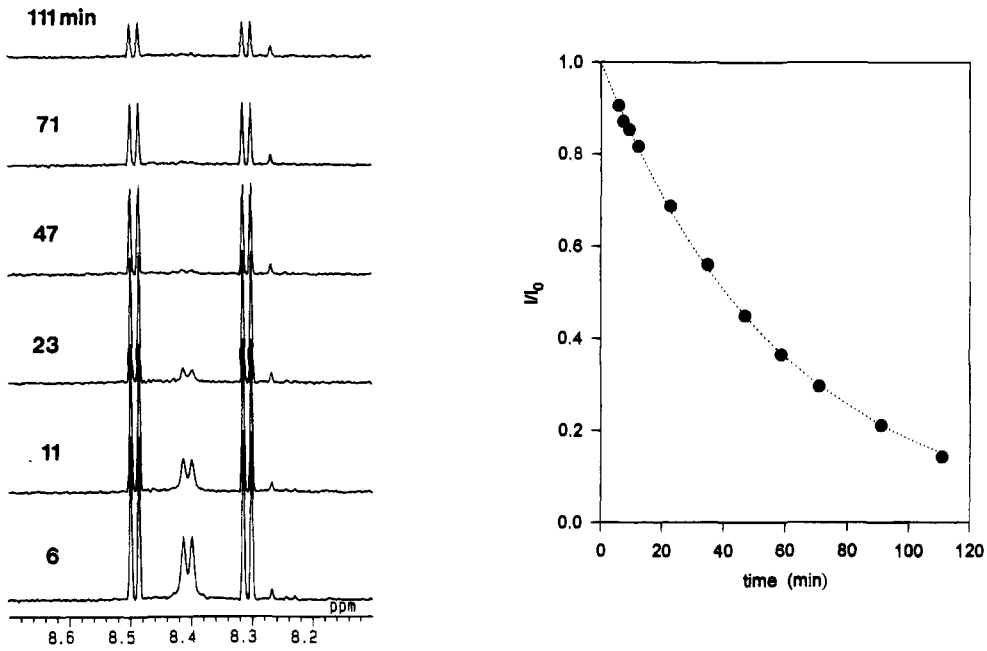

Figure 2. Time dependence of hydrogen–deuterium exchange of *N*-Ac-K-A-NH_2 in deuteriomethanol (2 mM sodium acetate), pH* 2.25, 20 °C. The A2 amide nitrogen is ^{15}N-labeled to separate its resonance from the K1 amide at 8.41 ppm (left). The time-dependent exchange data for the A2 amide are plotted with a least squares first-order fit (dotted line) on the right hand side.

with a tissue before measuring the pH* of the exchange sample after removal from the NMR tube into a 1.5 mL plastic microcentrifuge tube. While care must be taken in measuring accurate pH* values in methanol, the good fits of the pH*-dependent exchange data in this and previous studies[3] demonstrates that reliable pH* data can be obtained in this solvent. The estimated error in pH* measurements is around 0.1 pH* unit.[3a]

The decay of peptide amide signal intensity with time was fit to first-order decays using linear or semilogarithmic plots with Sigmaplot (Jandel Scientific) (Figure 2). pH-dependent exchange data were fit by a least squares algorithm to curves defined by eq 2 where k_{ex} is the

$$\log(k_{ex}) = \log(k_a[D^+] + k_b[OMe^-] + k_0) \quad (2)$$

experimental first-order exchange rate constant, k_a and k_b are the second-order rate constants for acid- and base-catalyzed exchange, and k_0 is the first-order rate constant for pH-independent exchange.[14] The concentration of base catalyst (OMe^-)[3a] was calculated using a value for the dissociation constant of methanol of $10^{-16.6}$.[15]

Values for pH^*_{min} and k_{min} (where pH^*_{min} is the pH* and k_{min} the exchange rate constant at the minimum of the pH-dependent exchange curves where $k_a[D^+] = k_b[OMe^-]$) were calculated from eqs 3 and 4, respectively[14].

$$pH^*_{min} = (1/2)[pK_{MeOD} - \log(k_b/k_a)] \quad (3)$$

$$k_{min} = k_a \times 10^{-pH^*_{min}} + k_b \times 10^{-(pK_{OMe}-pH^*_{min})} \quad (4)$$

Results

pH-Dependent Amide Exchange from Protected Dipeptides. Amide exchange from non-hydrogen-bonded amides in methanol has not been extensively characterized. To determine amide exchange protection factors that define the equilibrium constants for hydrogen bond fluctuations which limit amide exchange, a measure of the intrinsic exchange properties of non-hydrogen-bonded amides is required.[7,8] Previous studies of amide exchange from melittin in methanol used the exchange properties of the N-terminal amides (residues 3 and 4) which are known not to form hydrogen bonds in methanol[16] to determine exchange parameters (in particular k_{min}) for free amides.[3] Estimates of intrinsic k_{min} from these amides have possible errors arising from steric effects (because these amides, while not hydrogen bonded, lie at the N-terminus of a stable region of helix) and from uncertainties over the mechanism of acid-catalyzed exchange (see below). We have therefore measured the exchange properties of free amides in the protected dipeptide *N*-Ac-K-X-NH_2 where X is G, A, or Aib. This peptide was chosen for its high solubility in methanol, the absence of N- and C-terminal charges with large inductive contributions to amide exchange, and the absence of titratable groups over the pH* range of interest. We later found that *N*-Ac-A-A-NH_2 is sufficiently soluble in methanol for exchange measurements to be measured for this peptide also.

The pH* dependence of amide exchange for the protected dipeptides at 20 °C is shown in Figure 3, and the exchange parameters are listed in Table 1. Each peptide exhibits pH-dependent exchange rate constants that can be fitted to eq 2 with k_0 set to zero. The data for *N*-Ac-K-A-NH_2 and *N*-Ac-A-A-NH_2 give similar k_{min} values of 0.0068 and 0.0077 min^{-1}, respectively. These values can be compared with k_{min} for *N*-Ac-A-A-A-N-Me in water at 20 °C of 0.158 min^{-1}.[8] The ratios of the k_{min} values are 20–23, consistent with the lower concentration of exchange catalyst in CD_3OD ($K_{MeOH} = 10^{-16.6}$) compared with H_2O ($K_w = 10^{-14}$). [The expected decrease in k_{min} due only to the decreased exchange catalyst concentration is $10^{(16.6-14)/2}$ = 20-fold.] As previously suggested,[3a] this indicates that the slower exchange in methanol compared with water is largely due to the decreased total exchange catalyst concentration in methanol due to the suppressed ionization constant of this solvent.

The decrease of pH^*_{min} by around 1 pH unit in replacing the N-terminal A of *N*-Ac-A-A-NH_2 with K (Figure 3) results from the electrostatic effect of the K side chain positive charge which suppresses k_a by around 11-fold and enhances k_b by a similar amount. The observation that electrostatic[9] and inductive[8] effects have roughly equal and opposite effects on k_a and k_b for non-hydrogen-bonded amides is the basis for the use of k_{min}

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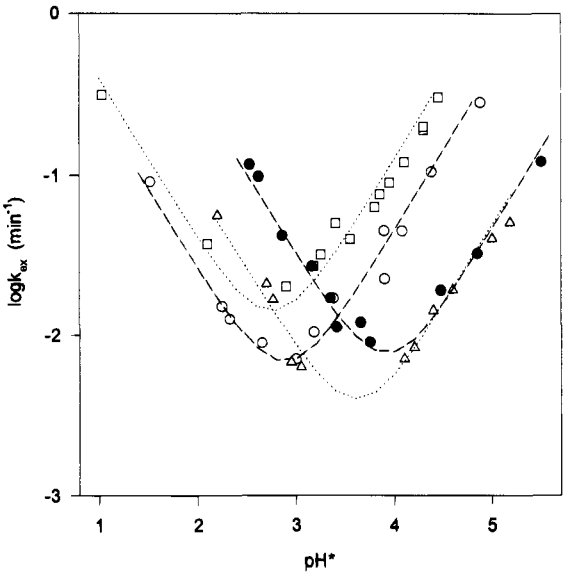

Figure 3. pH-dependent exchange rate constants for *N*-Ac-K-G-NH_2 (□), *N*-Ac-K-A-NH_2 (○), *N*-Ac-K-U-NH_2 (△), and *N*-Ac-A-A-NH_2 (●) in deuteriomethanol at 20 °C. The data belong to the amide of the underlined residue of the protected dipeptides. The dotted and dashed lines are fits of the experimental data to eq 2 with k_0 set to zero.

Table 1. Amide Exchange Parameters for *N*-Acetyl-K-X-amide and *N*-Acetyl-A-A-amide in Deuteriomethanol at 20 °C

X[a]	k_H (min^{-1})	k_{OMe} (min^{-1})	pH*$_{min}$	k_{min} (min^{-1})
G	4.0	5.0×10^{11}	2.75	0.0142
A	2.6	1.8×10^{11}	2.88	6.86×10^{-3}
Aib	8.18	1.95×10^{10}	3.61	4.0×10^{-3}
Ac-A-A-NH_2	32	1.85×10^{10}	3.92	7.7×10^{-3}

[a] The exchange parameters correspond to the peptide amide belonging to residue X.

values to determine amide exchange protection factors.[3] Protection factors based on the suppression of k_{min} relative to k_{min} for a non-hydrogen-bonded amide ($k_{min(free)}$) are expected to be independent of electrostatic and inductive effects because these effects alter pH_{min} without greatly affecting k_{min}. However, Bai *et al.* discovered significant sequence-dependent *steric* effects that cause suppression of both k_a and k_b for peptide amides on either side of bulky hydrophobic amino acids.[8] These effects probably cause the progressive 2-fold suppression of k_{min} in *N*-Ac-K-X-NH_2 from X = G to X = A to X = U (Figure 3). The small upward shift of pH_{min} (G to A) and the larger shift (A to U) presumably arise from the enhanced inductive effects of the addition of electron-rich methyl groups to the α-carbons of glycine and alanine.

We have used the limited data of Figure 3 and Table 1 to generate correction factors to k_{min} due to sequence-dependent steric effects for amide exchange in methanol. In line with the general trends described by Bai *et al.*[8] we have used k_{min} of *N*-Ac-A-A-NH_2 as the base value for exchange of A and make no additional correction to k_{min} for the amino acids T, S, D, E, Q, N, K, R, H, C, M, F, Y, W, and P, because the effects of these amino acids are largely inductive and do not greatly affect k_{min}.[8] The effect of G on its own amide is to enhance k_{min} relative to A by 2-fold (Figure 3), and the effect of Aib is to suppress k_{min} of its own amide by around 1.7-fold. We apply the same factor observed here for U to the amino acids V, L, and I which show sequence-dependent steric effects in exchange from amides in water.[8] While the major sequence-dependent steric effect is on the amide to the left of the side chain, there is a small effect on the amide on the right hand side.[8] We

Table 2. Sequence-Dependent Correction Factors to k_{min} Values for Exchange of Peptide Amides in Methanol

	L	R
group 1		
G	−0.32[a]	−0.16
group 2	0	0
A, T, S, C, M, P, E, Q, D, N, F, Y, W, H, K, R		
group 3	+0.23	+0.12
L, I, V, U		

[a] The correction factor is added to the logarithm of k_{min} for the amide to the left (L) or right (R) of the amino acid side chain.

therefore apply 1/2 of the correction factor to the amide on the right hand side of the amino acid in question. These approximate correction factors (to k_{min}) are defined in Table 2.

The values of k_{min} for *N*-Ac-K-A-NH_2 (6.8×10^{-3} min^{-1}) and *N*-Ac-A-A-NH_2 (7.7×10^{-3} min^{-1}) are significantly larger (by 4−5-fold) than the value previously used as $k_{min(free)}$ for non-hydrogen-bonded amides determined from amides near the N-terminus of melittin (1.6×10^{-3} min^{-1}). This difference has two likely origins. First, steric effects arising from a stable structure within which non-hydrogen-bonded amides exchange may suppress k_{min}. Such steric effects are observed in the suppressed exchange rate constants for poly-D,L-alanine (PDLA) compared with protected alanine di- and tripeptides[8] and the large suppression of k_{min} values for surface (non-hydrogen-bonded) amides of BPTI.[17] Second, any contribution from the imidic acid mechanism for acid-catalyzed exchange will suppress k_{min} for any amide in which the amide carbonyl is inaccessible.[10,18] These effects are considered further in the Discussion.

Measurement and Analysis of Amide Exchange Rate Constants for Alamethicin in Methanol. Davis and Gisin previously measured amide exchange rates in methanol-solubilized alamethicin using 600 MHz NMR spectroscopy in the correlation mode.[19] Their measurements were made at a single (unspecified) pH*, and the amide assignments were incomplete. Nevertheless, their results indicated high exchange stability for amides in the N-terminal helix. The good resolution of the amide signals (Figure 4) and the absence of titrating side chain groups (in the A6, Q18 version of the peptide used here) allowed extensive pH*-dependent data to be obtained in the present study.

Figure 4 also illustrates the potential errors in interpretation that may result from exchange measurements made at a single pH*. Several amides exchange considerably more slowly at lower pH* (acid-catalyzed exchange) (U1, U5, U13), whereas others (U17, Q18, Phol20) are much more stable toward base-catalyzed exchange. With some exceptions, amides near the C-terminus of the helical peptide and/or having relatively upfield shifted chemical shifts, are stable toward base-catalyzed exchange whereas those near the N-terminal end and/or having downfield chemical shifts are stable with respect to acid-catalyzed exchange. These effects are not accounted for by sequence-dependent inductive effects, but rather result from the fact that exchange of many of the amides occurs from native-like structures (so that structural effects on the amide chemical shift also influence the exchange properties) and that the effective dipole charges in helices have a significant influence on the exchange properties of the alamethicin amides (so that base-catalyzed exchange is suppressed for amides at the

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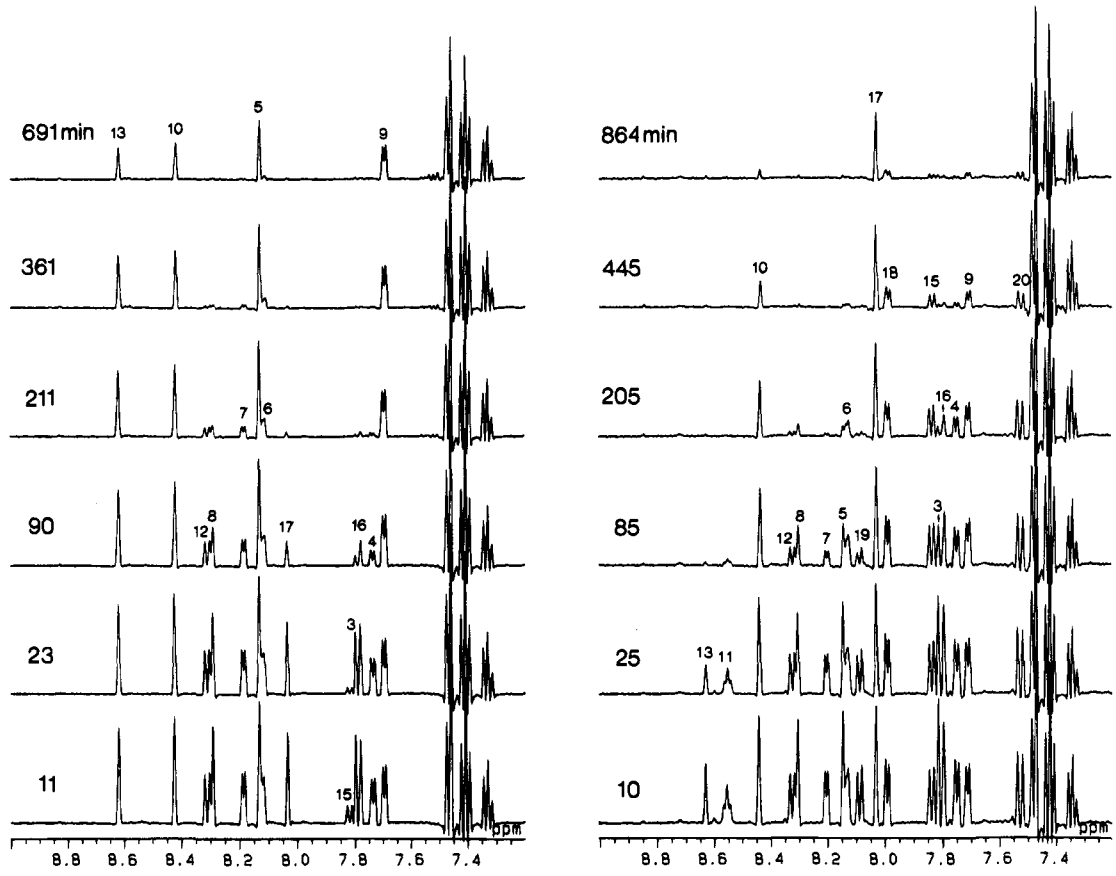

Figure 4. Time dependence of amide signal intensities (annotated with amino acid residue number) for alamethicin in deuteriomethanol at pH* −0.5 (left) and pH* 7.15 (right) at 20 °C.

C-terminus due to the C-terminal negative dipole charge). Correlations between amide exchange properties and the amide chemical shift were previously observed in apamin in water,[20] and the contribution of helix dipole charges to amide exchange in BPTI[17] and melittin[3] has also been noted.

The fits of pH*-dependent exchange data to eq 2 are shown in Figure 5, and the amide exchange parameters extracted from these data are listed in Table 3.

Exchange Protection Factors for Hydrogen Bond Breaking Backbone Fluctuations in Alamethicin. As observed for melittin,[3] amide exchange rate constants for alamethicin amides increase linearly with catalyst concentration at least up to pH* 8.0 (Figure 5), indicating that, over the pH* range investigated, exchange occurs in the so-called "EX_2" limit in which the fluctuation(s) limiting exchange is in preequilibrium with chemical exchange ($k_2 \gg k_3$). In the EX_2 limit, k_{ex}, the measured exchange rate constant, is equal to $K_{op}k_3$ (eq 1).

Values for the amide-specific protection factors (PF), corrected for inductive, electrostatic, and steric effects, provide the best estimate for K_{op} according to eq 1. PF values are determined here in two ways and are listed in Table 4 along with similar data for melittin calculated from data in ref 3. One set of PF values, designated r_{min}, is based on the suppression of k_{min} relative to k_{min} for *N*-Ac-A-A-NH_2 ($k_{min(free)}$), corrected for the sequence-dependent steric effects of Table 2 (*i.e.*, $r_{min} = k_{min(free)}/k_{min(c)}$ where $k_{min(free)}$ is k_{min} for *N*-Ac-A-A-NH_2 and $k_{min(c)}$ is k_{min} for the amide of interest, corrected for sequence-dependent steric effects). Second, separate PF values for acid- and base-catalyzed exchange (PF_a and PF_b, respectively) are determined by calculating the suppression of the rate constant, k_a or k_b, relative to k_a or k_b for *N*-Ac-A-A-NH_2 (Table 2) and correcting for sequence-dependent inductive and steric effects using the correction factors of Bai *et al.*[8] In this calculation it is assumed that these sequence-dependent inductive effects measured for exchange in water are similar to the inductive contributions of amino acid side chains to exchange in methanol although this remains to be fully investigated. The steric and inductive effects of Aib are taken from the data of Table 2.

The protection factors for alamethicin amides in methanol are illustrated graphically in Figure 6 as a function of the amino acid residue. Whether the protection factors are based on r_{min} values (Figure 6A) or corrected base-catalyzed exchange rates (Figure 6B), all the alamethicin amides (except U1) show suppressed exchange, indicating their protection within hydrogen bonds. The base-catalyzed protection factors (PF_b) are generally smaller than PF values measured from r_{min} values except for the C-terminal amides, and this reflects the contribution of the acid-catalyzed PF values [which are generally larger than base-catalyzed PF values (Figure 6C)] to PF values measured from k_{min}.

Discussion

Characterization of the properties of monomeric alamethicin in methanol in terms of the stability of individual hydrogen bonds using amide exchange analysis requires measurement of exchange protection factors that isolate the effects of hydrogen bonding from other influences on amide exchange. Although the main conclusions, described below, concerning the hydrogen bonding pattern and relative hydrogen bond stabilities in alamethicin are largely independent of the method of calculating protection factors, the factors based on suppression of k_{min} are generally larger than protection factors based on the suppression of k_b alone. Determination of the best estimates of K_{op} values

(20) Dempsey, C. E. *Biochemistry* **1986** *25*, 3904−3911.

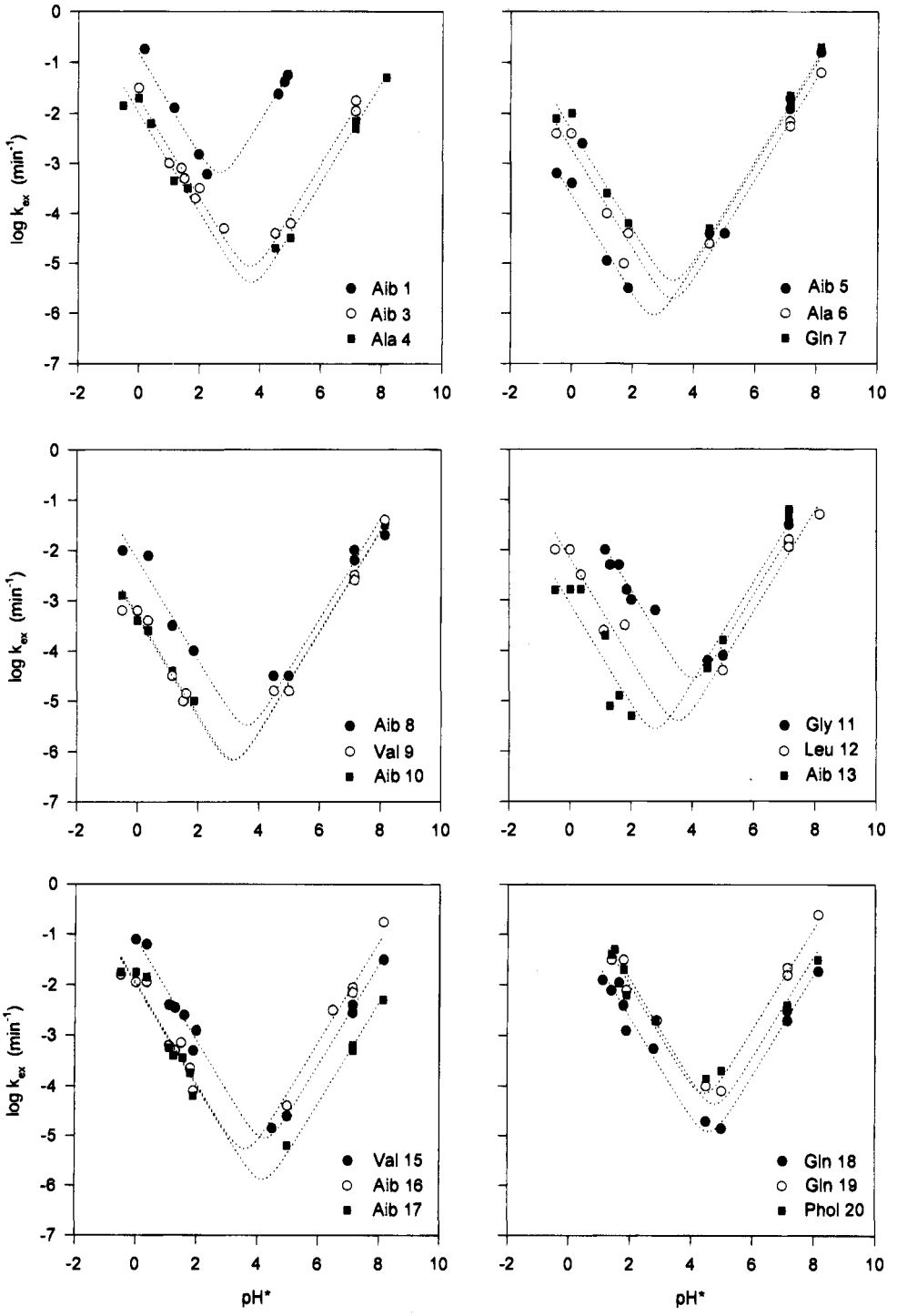

Figure 5. pH-dependent exchange rate constants for alamethicin amides in deuteriomethanol at 20 °C. Dotted lines are fits to eq 2 with k_0 set to zero.

yields an optimal description of local polypeptide backbone stability, and these measures can be used to determine the influence of amino acid substitution and other factors on hydrogen-bonded structure and stability[3b,21] and to make quantitative comparisons between experimental and theoretical data.[4-6] The first section of the discussion deals with the determination of exchange protection factors for polypeptides in methanol and the second with a description of the structural and dynamic properties of the alamethicin helix and the effects of the P14 residue, determined from the exchange analysis.

Determination of Exchange Protection Factors in Methanol. Helix Dipole Effects. The use of k_{min} values to estimate K_{op} for hydrogen bond fluctuations limiting amide exchange has been described before.[3b] With the assumption that all inductive and electrostatic effects influence k_a and k_b to roughly equal and opposite extents, these effects are minimized in averaging uncorrected acid- and base-catalyzed exchange protection factors. This approximate reciprocality of inductive effects on the acid- and base-catalyzed exchange rate constants of unstructured peptides is seen in the exchange data of Bai *et al.*,[8] where most amino acid substitutions shift pH_{min} without greatly affecting k_{min}, and this reciprocality is expected to apply to generalized electrostatic effects on the acid- and base-catalyzed rate constants.[9] This leaves only the effects of

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Table 3. Amide Exchange Parameters for Alamethicin in Methanol at 20 °C

residue	k_H (min^{-1})	k_{OMe} (min^{-1})	pH*$_{min}$	k_{min} (min^{-1})
U1	0.150	2.80×10^{10}	2.67	6.50×10^{-4}
P2				
U3	0.0202	3.74×10^{7}	3.67	8.76×10^{-6}
A4	0.0103	1.65×10^{7}	3.70	4.10×10^{-6}
U5	2.30×10^{-4}	3.71×10^{7}	2.70	9.00×10^{-7}
A6	1.96×10^{-3}	1.95×10^{7}	3.30	1.90×10^{-6}
Q7	4.70×10^{-3}	4.36×10^{7}	3.32	4.50×10^{-6}
U8	6.50×10^{-3}	1.69×10^{7}	3.59	3.30×10^{-6}
V9	4.6×10^{-4}	9.77×10^{6}	3.14	6.0×10^{-7}
U10	5.0×10^{-4}	9.33×10^{6}	3.16	6.0×10^{-7}
G11	0.153	5.1×10^{7}	4.04	2.80×10^{-5}
L12	6.8×10^{-3}	2.37×10^{7}	3.53	4.0×10^{-6}
U13	8.5×10^{-4}	9.18×10^{7}	2.78	2.7×10^{-6}
P14				
V15	0.0803	9.92×10^{6}	4.25	8.9×10^{-6}
U16	0.011	2.75×10^{7}	3.60	5.5×10^{-6}
U17	0.010	1.71×10^{6}	4.19	1.3×10^{-6}
Q18	0.238	6.33×10^{6}	4.59	1.23×10^{-5}
Q19	1.02	5.16×10^{7}	4.45	7.25×10^{-5}
Phol20	1.34	1.45×10^{7}	4.79	4.41×10^{-5}

Table 4. Amide Exchange Protection Factors for Alamethicin and Melittin (MEL) in Methanol (20 °C)

residue	PF(r_{min}[a])	PF(k_b(Bai)[b])	residue (MEL)[c]	PF(r_{min})	PF(k_b(Bai)
U1	6.3	0.046	G1		
P2			I2	3	0.1
U3	480	140	G3	6.6	0.4
A4	1300	560	A4	4.6	2.8
U5	4600	55	V5	65	7.3
A6	2750	470	L6	nd[d]	nd
Q7	1500	490	K7	nd	nd
U8	1300	190	V8	92	20
V9	5100	190	L9	63	9
U10	5100	160	T10	73	55
G11	400	290	T11	160	370
L12	1450	300	G12	23	29
U13	1150	31	L13	24	4
P14			P14		
V15	440	210	A15	18	4
U16	580	52	L16	140	11
U17	2400	590	I17	1100	81
Q18	430	1650	S18	590	200
Q19	90	650	W19	1050	130
Phol20	160	650	I20	2800	340
			K21	1150	900
			R22	600	1150
			K23	200	300
			R24	60	50
			Q25	14	60
			Q26	9	120

[a] The ratio of k_{min} for *N*-Ac-A-A-NH$_2$ divided by k_{min} for each amide, corrected for the sequence-dependent contributions to k_{min} of Table 2. [b] Suppression of k_b for each amide relative to k_b for *N*-Ac-A-A-NH$_2$ corrected for sequence-dependent inductive and steric contributions to k_b of Bai *et al.*[8] [c] Calculated from the data in ref 3a. [d] Not determined.

hydrogen bonding and steric effects (which suppress both k_a and k_b) as influences on k_{min}. When sequence-dependent steric effects are minimized (using, for example, the data of Table 2), and neglecting potential conformation-dependent steric effects on exchange that cannot currently be measured (because the nature of the "open" state from which exchange occurs is poorly characterized), the protection factor measured from the suppression of k_{min} relative to k_{min} for a non-hydrogen-bonded peptide amide is expected to be a reasonable estimate for $1/K_{op}$.

One advantage of the use of k_{min} values is the potential for accounting for conformation-dependent electrostatic contributions to exchange. Peptide helices have an associated macrodipole equivalent to ±0.5−0.75 unit charge at the N- and C-termini which is maximal close to the helix termini in the direction of the helix axis.[22] The helix dipole charge can potentially influence amide exchange by suppressing (enhancing) acid- (base-) catalyzed exchange at the N-terminus with the opposite effect at the C-terminus. These effects are the likely cause of the large enhancement (by 20-fold) of the base-catalyzed exchange rate constant for U1 of alamethicin and the shift of pH*$_{min}$ to high pH for amides at the C-terminus of alamethicin (Figure 5, Table 3) and melittin.[3] The variant of alamethicin used here has no side chain or helix termini formal charges, and the large shifts of pH*$_{min}$ to higher pH* for the C-terminal -Q-Q-Phol tripeptide by up to 1.3 pH* units, together with similar effects observed in melittin,[3] must arise largely due to the effects of the helix dipole. The effects of helix dipole charges on amide exchange have been observed in other cases[3,17] (see also ref 4). The large factors observed here (around 15−20-fold, resulting in shifts of pH*$_{min}$ by 1.2−1.3 pH* units for helix terminal amides) are due to the enhancement of electrostatic contributions in lower polarity solvent (methanol compared to water) and the low ionic strength of the methanolic solution. Because the helix dipole effects are minimized in combining acid- and base-catalyzed protection factors, the data of Figure

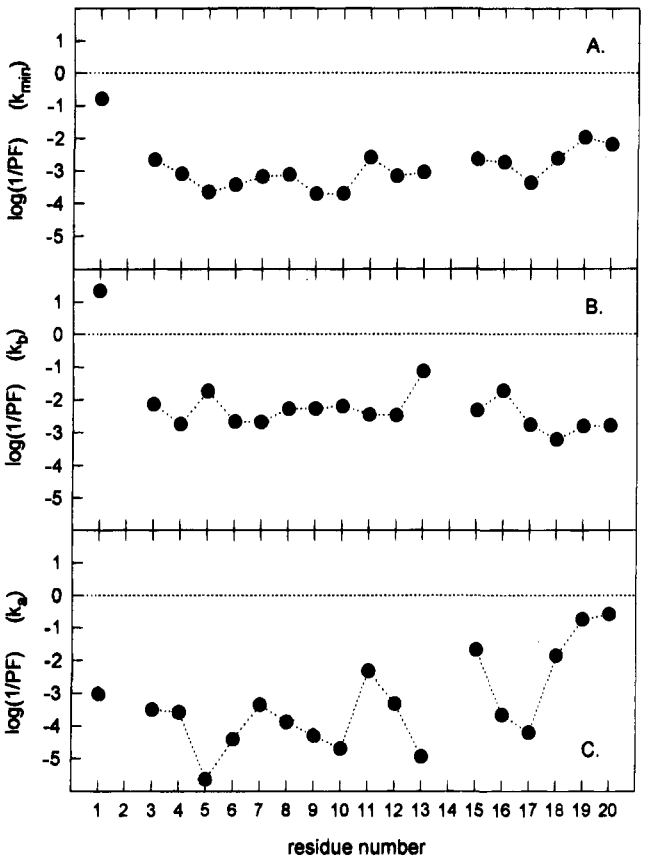

Figure 6. Logarithms of the protection factors for exchange of alamethicin amides in methanol (20 °C) as a function of amino acid residue relative to *N*-Ac-A-A-NH$_2$: (A) protection factors (PF) based on suppression of k_{min} corrected for the sequence-dependent steric effects of Table 2; (B) PF values based on suppression of base-catalyzed exchange rate constants corrected for sequence-dependent inductive and steric effects;[8] (C) PF values based on suppression of acid-catalyzed exchange rate constants corrected for sequence-dependent inductive and steric effects.[8] In each case the dotted line indicates a protection factor of zero determined from the relevant exchange parameter (k_{min}, k_b, or k_a) for *N*-Ac-A-A-NH$_2$. The data for panels A and B are estimates of K_{op} (=1/PF) for hydrogen bond openings according to eq 1.

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6A are probably more accurate for the C-terminal amides of alamethicin than those based on corrected base-catalyzed protection factors (Figure 6B) which suggest that hydrogen bonds involving the C-terminal amides (of residues Q18, Q19, and Phol20) are among the most stable in the alamethicin helix (contrary to evidence from NMR structural analysis of the peptide in methanol[12]).

Acid-Catalyzed Exchange. The most serious limitation to the use of k_{mln} data for determining exchange protection factors for estimating K_{op} is the contribution of the imidic acid mechanism for acid-catalyzed exchange. Perrin and co-workers have presented strong evidence that acid-catalyzed exchange from polypeptides occurs *via* protonation at the amide carbonyl (eq 5) rather than the amide nitrogen (eq 6) with a transition

$$\mathrm{RCONHR'} + \mathrm{H^+} \rightleftharpoons \mathrm{RC(OH){=}NHR'^+} \rightleftharpoons \mathrm{RC(OH){=}NR'} + \mathrm{H^+} \quad (5)$$

1

$$\mathrm{RCONHR'} + \mathrm{H^+} \rightleftharpoons \mathrm{RCONH_2{}^+R'} \quad (6)$$

state resembling the imidic acid (**1**).[10] Exchange *via* this mechanism requires that the amide carbonyl is accessible for protonation, indicating that hydrogen bonds protecting *both* the amide nitrogen *and* the amide carbonyl must be broken for exchange to occur.[10] Evidence for the imidic acid mechanism in acid-catalyzed exchange from polypeptides has been obtained for surface amides in BPTI[18] and for the amides in a helical alanine-based peptide.[4] Because of the requirement for freeing the amide carbonyl and the amide nitrogen from hydrogen bonds, a major contribution from this mechanism is expected to cause greater suppression of acid-catalyzed exchange relative to the suppression of base-catalysed exchange.[10a] Enhanced acid-catalyzed protection factors are observed for alamethicin (Figure 6B,C), indicating that the imidic acid mechanism is probably important for acid-catalyzed exchange in this peptide. Apart from the C-terminal amides whose suppression (enhancement) of base- (acid-) catalyzed exchange rate constants results from effects of the helix macrodipole and the amides of Aib residues 5, 13, and 17 (which have greatly suppressed acid-catalyzed exchange[23]), the acid-catalyzed protection factors are around 5−50-fold greater than base-catalyzed rate protection factors. Generally, the acid-catalyzed PF values are less than the product of the base-catalyzed PF values of the relevant amides (*i.e.*, amides of residues i (NH) and $i + 3$ (CO) for an α-helix) expected if exchange from alamethicin in methanol occurred by small scale hydrogen bond openings in which both the amide nitrogen and carbonyl were exposed simultaneously only as the product of independent individual hydrogen bond openings. This reflects the cooperativity of hydrogen-bonded structure (so that during some fluctuations, hydrogen bonds freeing the amide NH and carbonyl components will open concertedly), and the possibility that acid-catalyzed exchange may revert to the N-protonation mechanism for amides in which the carbonyl component is inaccessible.[24] Determination of better estimates of acid- and base-catalyzed exchange protection factors through the measurement of more extensive sequence-dependent exchange data from model peptides may allow this approach to be used to determine the extent to which fluctuations freeing amides for exchange involve cooperative opening of secondary structure (in which case acid- and base-catalyzed exchange protection factors will be equal) or by more local opening of individual hydrogen bonds as expected, for example, in helix "fraying" where acid-catalyzed protection factors are expected to be suppressed relative to base-catalyzed protection factors.[4]

Because of the effects of imidic acid exchange chemistry on acid-catalyzed exchange and the combination of acid- and base-catalyzed exchange rate constants in protection factors based on k_{min} (Figure 6A), the latter protection factors are somewhat enhanced (excluding at least the C-terminal di- or tripeptide whose amide carbonyls are always free) and K_{op} values will be artificially suppressed. Some uncertainty remains therefore over the optimum method for determining exchange protection factors for hydrogen-bonded amides in methanol, and a satisfactory resolution may require a more extensive set of data from model unstructured peptides to determine sequence-dependent inductive contributions to exchange in methanol similar to those available for exchange in water.[8] In general it seems preferable to measure both acid- and base-catalyzed exchange rate constants so that electrostatic influences on exchange may be estimated, because these are enhanced in methanol and the use of high salt concentrations for charge screening is inappropriate in this solvent. In analyzing hydrogen-bonded structure and stability in alamethicin, in the following section we base our discussion on PF values measured from the suppression of k_{min} (Table 3, Figure 6A) because these values are little affected by inductive and electrostatic effects, and can readily be compared with exchange data from melittin. Because k_{min} values for amides N-terminal to Q18 may be additionally suppressed due to the contribution of imidic acid exchange chemistry, we take a conservative approach in interpreting exchange data in terms of hydrogen bond stabilities, and our conclusions are equally accommodated by PF values based on k_b corrected for sequence-dependent steric and inductive effects (Figure 6B).

Hydrogen-Bonded Structure and Stability of Alamethicin in Methanol. The variant of alamethicin studied here has eight Aib residues. The conformational restrictions induced by the replacement of the CHα proton of alanine with a methyl group in Aib severely restrains rotations around the adjacent N−Cα and Cα−C′ bonds (defined by the ϕ and ψ torsion angles, respectively), and the allowable torsion angles are restricted to narrow regions corresponding to α-helix or 3_{10} helix geometry.[25] The incorporation of Aib residues into polypeptide sequences composed of L-amino acids strongly stabilizes right-handed helical structure. It is not surprising, therefore, that alamethicin has high helix stability. It is remarkable however that the P14 residue does not cause a major disruption of helical hydrogen bonding at any amino acid residue. In melittin (Figure 1), the presence of P14 (together with G12) causes a large destabilization of α-helical structure involving the amides of residues 15, 13, and 12 so that hydrogen bonding in one helical turn is disrupted (Table 4) and the N- and C-terminal helical sections are connected by a flexible helical bend.[3,16,26] In an α-helix the distortions required to accommodate an internal proline normally result in the loss of hydrogen bonding of the residue following P because the hydrogen distance (NH_{i+1} to $C{=}O_{i-3}$ where i is P) becomes too long for an acceptable hydrogen bond.[27] The high exchange stability of V15 NH in alamethicin

(23) This may be partly due to unaccounted-for inductive or electrostatic contributions to exchange for these amides because base-catalyzed exchange is enhanced relative to adjacent amides.

(24) Acid-catalyzed exchange from peptide amides adjacent to Aib may already occur largely by N-protonation (eq 6) due to the electron-donating properties of the additional α-methyl group which is expected to increase the basicity of the amide nitrogen, favoring the N-protonation mechanism.[10]

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indicates that this amide remains hydrogen bonded, probably within a 3_{10} helical hydrogen bond (with the L12 carbonyl as the hydrogen bond acceptor) as is observed in the crystal structure.[13a] A 3_{10}-type helical hydrogen bond probably accounts for the high exchange protection of U3 (with the *N*-acetyl carbonyl as the hydrogen bond acceptor). Analysis of atom trajectories extracted from 100 ps molecular dynamics of alamethicin "solvated" in methanol indicates that these 3_{10} hydrogen bonds are favored in alamethicin (Williams, P. B.; Sessions, R. B.; Harvey, A.; Dempsey, C. E. Submitted for publication). The high-resolution NMR structure of alamethicin in methanol shows that the peptide is α (rather than 3_{10}) helical at least through the N-terminal helix.[12a] Reexamination of NOEs from alamethicin in cold methanol to stabilize the structure and enhance NOE intensities supports this conclusion; of five possible $CH_\alpha-NH_{i+4}$ NOEs characteristic of an α-helix over a 3_{10} helix in the N-terminal 13-residue section, two are unobservable because of resonance overlap, but two are observed ($A4_\alpha-U8_{NH}$ and $A6_\alpha-U10_{NH}$), indicating that alamethicin is α-helical (rather than 3_{10} helical) in the residues preceding P14 (unpublished results). It is likely (and consistent with molecular dynamics simulations) that small deviations from α-helical geometry allows 3_{10} hydrogen bonding that results in the exchange protection of V15 and U3 NH's. While some uncertainty remains over the structure of the C-terminal residues,[12] the high exchange stabilities of amides up to Phol20 NH indicates that the peptide is helical throughout. The exchange data are consistent with the hydrogen bonding pattern observed for alamethicin crystallized from methanol by excess acetonitrile,[13a] except that in the crystal structure the amide NH's of residues U16 and U3 do not form hydrogen bonds.

Every amide NH of alamethicin except U1 has at least 30-fold suppression of amide exchange, indicating stabilization by hydrogen bonding (Figure 6A,B). The high helix stability of alamethicin with respect to backbone fluctuations that break hydrogen bonds is consistent with the results of ^{13}C NMR relaxation measurements,[12b] indicating a low contribution of backbone fluctuations on the nanosecond time scale to relaxation, even around P14. Although fluctuations occurring on a time scale that makes little contribution to NMR relaxation (such as slow helix flexing around P14) are not incompatible with the ^{13}C relaxation data, the amide exchange data demonstrate that these fluctuations occur rarely at least to the extent that they result in transient hydrogen bond disruption. The data demonstrate that, in a medium where intramolecular hydrogen bonding is favored, the alamethicin helix is stable with respect to major backbone fluctuations that allow amide exchange to occur. The exchange data are therefore more consistent with models for voltage activation of "prepore" states of alamethicin involving reorientation of stable helices within the membrane,[28] rather than those involving voltage-dependent recruitment of disordered C-terminal structure into a helix.[13] Amide exchange measurements from membrane-reconstituted alamethicin support this conclusion in showing heavily suppressed exchange for all the potentially hydrogen-bonded amides including those of residues near, and on the C-terminal side of, P14 (Dempsey, C. E.; Handcock, L. J. Submitted for publication).

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