

Determination of the Barriers to Exchange and Temperature Stability of a Helical Peptide Using NMR Spectroscopy

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Increasingly, disorder and motion are being shown to play an important role in protein function. We are investigating the motions of helices using short chains of the sterically hindered amino acid Aib (α -methylalanine) as models. Peptides rich in Aib have been shown to adopt 3_{10} -helical structures whose stabilities depend on chain length and amino acid composition. Their dynamics are assessed by examining the rate of amide proton-deuteron exchange due to frequent interactions with the solvent (deuteron donor). Regions that remain relatively rigid experience slow exchange, while those that are flexible should exchange more quickly. Preliminary results indicate that while the C- and N- terminal regions of shorter peptides (4-6 amino acids) are more dynamic than the centers, the C-terminal regions of peptides 8 amino acids in length are significantly less disordered. Temperature-dependent studies of the hydrogen-deuterium exchange in a hexamer are presented here to determine the activation energies for the exchange of the amide protons.

INTRODUCTION

Proteins are amino acid polymers that perform many functions including enzymatic catalysis, transportation of materials in the blood, and transport of chemical messages within the endocrine system and across membranes (1). The structure and stability of proteins determines their biological behavior, and thus gives information about how function of that particular molecule is performed. The sequential order of amino acids in a protein or peptide is called primary structure. Some peptides are able to fold into secondary structures, one type of which is the helix (2). The overall stability of helical peptides can be analyzed by comparing the strength of the intramolecular hydrogen bonds formed between the amide and carbonyl functional groups along the helix, which help to stabilize the helices substantially.

The Aib (α -methylalanine) residue (Figure 1) is a naturally occurring amino acid that is not one of the twenty common amino acids. It is found in some bacterial channel-forming proteins in which Aib accounts for 30-50% of the residues (3). Aib is dialkylated at the α -carbon, providing a great deal of steric hindrance. This hindrance strongly biases Aib peptides towards helical structures (4, 5). Short peptides composed entirely of Aib can form 3_{10} -helices. 3_{10} -helices are interesting because they are rare, accounting for about 10% of helices found in protein structures (2). Furthermore, they are thought to act as intermediates in the α -helix folding pathway (6, 7), and may be involved in the function of some proteins (8, 9). Thus, Aib-containing peptides provide ideal models with which to investigate the stabilization effects of 3_{10} -helical structures (10).

Information on the strength of the hydrogen bonds and the stability of the overall helix structure can be gained from the rate of exchange of amide protons with deuterons from the

solvent. In this case, the relative strength of each hydrogen bond and the accessibility of protons to the solvent are characterized in terms of their rate constants for exchange and the activation barriers to exchange using pseudo first order kinetics. The intramolecular hydrogen bonding to an amide proton provides an additional barrier to exchange (increases the activation energy) beyond that of a free N-H experiencing exchange. Accessibility to the solvent is a diagnostic of the flexibility of the helix at a particular area and indicates the amount of solvent interaction at that site on the helix. Sites with high accessibility are likely to exchange faster than those that are not accessible (have a decreased collision frequency). The activation energy and collision frequency can be obtained from studying the effect of temperature on the rate of exchange. Previous studies have shown that Aib peptide helices are stable at temperatures up to at least 373 K (11). In this study, the hexameric peptide tBoc-B₆-FI, is analyzed by obtaining ¹H NMR spectra on peptides dissolved in a solvent containing an exchangeable deuteron (CD₃OD) over a temperature range of 303–318 K.

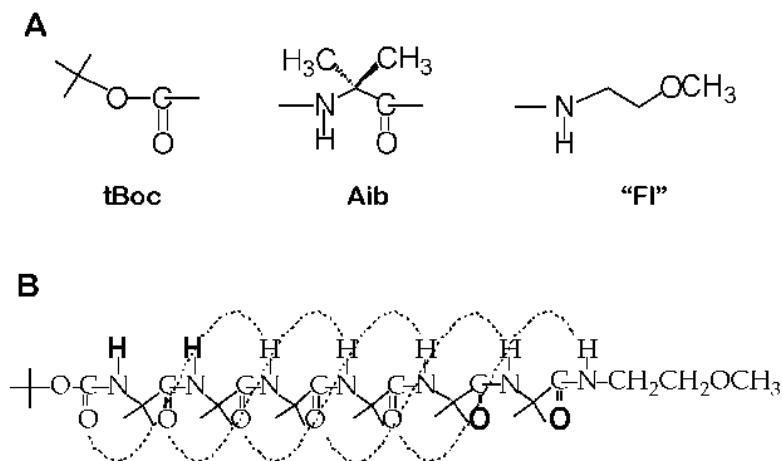


Figure 1. Structure of the 3_{10} -helical peptide tBoc-B₆-FI, where "B" represents an Aib amino acid residue. A. The building blocks of tBoc-B₆-FI. This peptide is soluble in organic solvents because of the end groups that are on the C-terminus (tBoc) and N-terminus (FI) of the helix. B. Demonstration of the way that the amide protons are hydrogen bonded to the carbonyl group of the third amino acid down the chain (3_{10} -helical hydrogen bonding pattern).

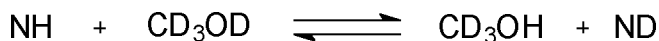
METHODS

Peptide samples were dissolved in a 1% CDCl₃/CD₃OD solution at a concentration of approximately 10 mM. The deuterated methanol was used as the exchange solvent and the deuteriochloroform was used for spectral resolution of the NH peaks from each other. NMR spectra were obtained on a Bruker 300 MHz NMR spectrometer at temperatures of 303, 308, 313, and 318 K. A 45° pulse angle, rather than 90°, was used to ensure that the magnetization vector was allowed to return to equilibrium before it was pulsed by the magnetic field again. This was done also because of the fact that the rate of exchange (using kinetic modeling) is time dependent. Using a 90° pulse would have meant that the time for the relaxation back to

equilibrium would have been longer, and thus more exchange would have occurred during the measurements. Timing of the exchange reaction began as soon as the solvent was added to the peptide. The sample was allowed to equilibrate to the appropriate temperature for approximately five minutes prior to acquisition. The exchange processes were observed for a time period long enough (at least 300 minutes) to see significant decay of the integrated peak areas. The data obtained on the Bruker was transferred to a PC for processing.

Processing was performed using the WinNuts software package. After Fourier transformation and phasing, the baseline was corrected (for DC offset and/or tilt) and then the integral phasing was readjusted to make sure that the integrals were parallel with the baseline. To ensure similar integration throughout a given experimental run, the subsequent spectra from the same temperature experiment were processed using the exact same baseline and phasing adjustments. Chemical shifts were referenced to TMS (tetramethylsilane) at 0 ppm. The integrated NH peak areas were determined relative to that of the Aib methyl peaks, which do not exchange with the deuteron in the solvent system.

The pseudo first order kinetics studies were carried out under flooded conditions, where an excess concentration of the solvent is used so that the solvent concentration can be treated as approximately constant. This is done so that the general reaction equation can be simplified to a first order reaction with a pseudo first order rate constant. The general exchange reaction equation for each amide is:



where equilibrium is pushed far to the right because of excess CD_3OD . Using this general equation, the rate law for exchange of each amide is given by:

$$\text{Rate} = k [\text{NH}][\text{CD}_3\text{OD}] \quad (1)$$

$$\text{with } k' \approx k [\text{CD}_3\text{OD}] \quad (2)$$

since the solvent is flooded. Thus, the pseudo first order rate law is given by:

$$\text{Rate} = k' [\text{NH}] \quad (3)$$

where the relative peak area under each NH peak represents the concentration of that amide proton. This concentration was plotted against time according to equation 4 (an integrated form of equation 3):

$$\ln(I) = \ln(I_0) - k't \quad (4)$$

where I is the relative area (concentration) of a given NH peak, I_0 is the concentration at $t=0$, k' is the pseudo first order rate constant, and t is the time at which the peak area was measured.

The activation energy for each NH was determined from a linear plot of the Arrhenius equation,

$$\ln(k') = \ln(A) - E_a/R(1/T) \quad (5)$$

where A is the collision frequency, E_a is the activation energy, R is the ideal gas constant, and T is the temperature in K. Estimates of the collision frequency contained too much error to be reported reliably.

All linear fits were performed using Linear Least Squares (LLS) analysis (using Microsoft Excel spreadsheets). Errors are reported as standard deviations from the fit.

RESULTS

The tBoc-B₆-Fl peptide folds into a helix that contains two different types of amide hydrogens. Group 1 (B1 and B2) has hydrogens that are not intramolecularly hydrogen bonded to

the carbonyls of other amides in the helix (see Figure 1: bolded H). Group 2 contains those amides that are intramolecularly hydrogen bonded to other carbonyls (B3, B4, B5, B6, and FI). The NMR spectrum of the amide region of tBoc-B₆-FI as a function of time is shown in Figure 2.

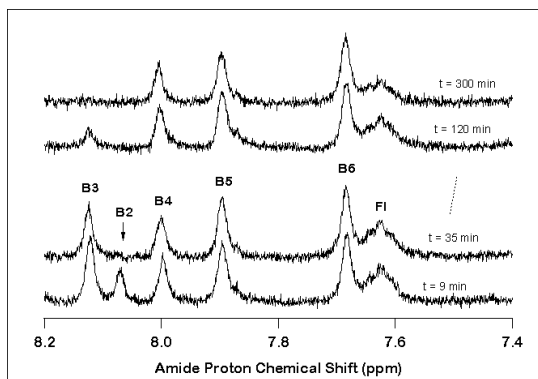


Figure 2. ¹H NMR spectra of the amide region of tBoc-B₆-FI as a function of time (308 K). The plot indicates the decay in the concentration of the amide protons (area of the peaks) as they are exchanged for deuterons.

From Figure 2, it is evident that as time progresses, the amide proton signals decay. Furthermore, the Group 1 protons exchange at a much more rapid rate than the Group 2 protons. The Group 1 proton B1 exchanges almost completely before the first scan was obtained, and thus is not observed in these spectra. The other amide in this group, B2, also exchanged rapidly, indicated by the decay in signal in a minimal amount of time (less than 35 minutes). The hydrogen bonded amide B3 showed the greatest decay in signal relative to the rest of the protons in Group 2. As a whole, the hydrogen bonded protons (Group 2) had a dramatically slower decay in signal as compared to that for the Group 1 protons.

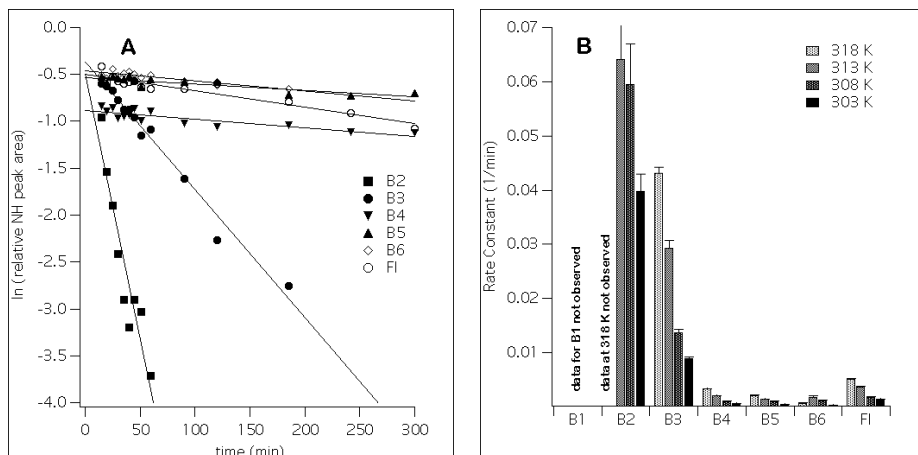


Figure 3. Pseudo first order kinetics plots. A. Pseudo first order rate law plots at 308 K. The rate constants are obtained from the slopes of the lines. B. Effect of temperature on exchange rate constants. Amides at the N-terminus exchange faster than those at the center or even those at the C-terminus of the helix, suggesting greater stability near the center and the C-terminus of the helix.

Pseudo first order plots (Figure 3A) of equation 4 were used to determine the rate constant for exchange at a given temperature. The slope of the line is the indication of the pseudo first order rate constant. The larger the rate constant, the steeper the line, and thus the faster the exchange is taking place. The intercept is related to the concentration of the amide proton at the beginning of the experiment. The intercepts are not equal to zero because the initial relative area of all amide protons were not equal to one. This is because the deuterons may not have completely exchanged back to protons from the previous exchange experiment. This does not affect the results presented here because only the change in concentration is relevant. The use of pseudo first order kinetics to model the exchange reaction is justified by the quality of the fits in Figure 3A.

The pseudo first order rate constants for exchange are obtained from the LLS fits to equation 4, for each respective proton, and are plotted in Figure 3B. This figure supports the observations that were seen in Figure 2, that B2 exchanges very quickly, B3 exchanges moderately quickly, and B4-FI exchange very slowly. This shows that the hydrogen bonded protons exchange at a slower rate than the protons that lack this hydrogen bond. The proton for B1 exchanged almost entirely during the time that was allowed for the sample to equilibrate with the instrument temperature, therefore values for k' were not obtained for this amide proton. Also, B2 exchanged too quickly to be observed when the temperature was elevated to 318 K, and had a high rate constant when the temperatures were slightly lower than 318 K. The hydrogen bonded B3 amide proton also had high rate constants as compared to the remaining hydrogen bonded protons in the helix. In fact, the rate of exchange for B3 at 318 K was approximately the same as that of B2 at the temperature of 303 K. This shows that B3 is exchanging much more rapidly than the rest of the hydrogen bonded amides.

The rate of the exchange is dependent upon the temperature at which the reaction takes place. The data seen in Figure 3B shows that as the temperature was decreased, the rate constant for each amide proton also decreased. This trend was not observed for B6 because of the interference caused by the splitting pattern of the FI group. This FI group has a wider

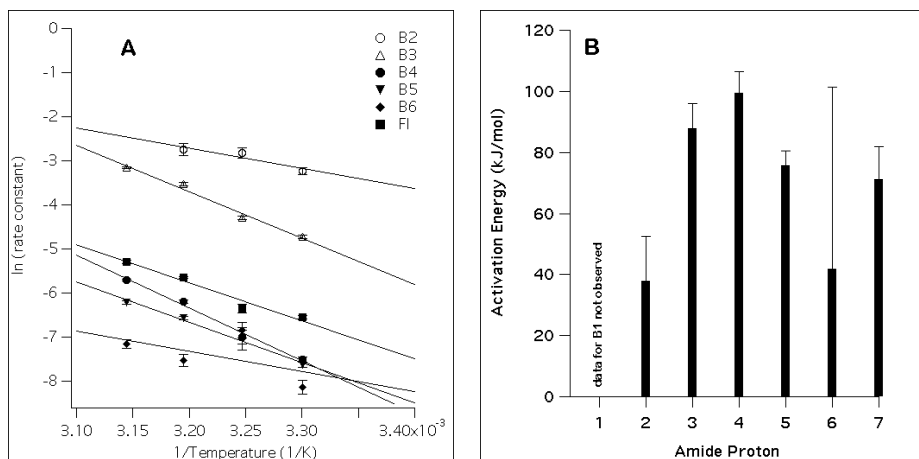


Figure 4. Effects of temperature on the exchange of amide protons. A. Arrhenius plots for the amide protons in tBoc-B₆-FI. B. Activation energies for exchange of the amide protons. The activation energies appear to be similar for those amides that have intramolecular hydrogen bonding.

peak area than any of the other amide proton peaks because of the splitting pattern from a neighboring CH_2 group, and thus there is overlap in the integral areas of B6 and F1 (Figure 2). The exchange rate of these two protons is different (Figure 3), and thus integration of each peak yields a contribution from the exchange of both peaks together. This means that the error is dramatically enhanced for the proton that exchanges more slowly (B6) because there is some influence in the exchange caused by the slightly faster exchanging proton with overlapping integral areas.

Arrhenius plots of the rate constants for exchange as a function of time for each amide proton were used to obtain the activation energies for exchange (Figure 4A). The slope is an indication of the activation energy, which are plotted in Figure 4B for each amide proton. Activation energies are similar for the hydrogen-bonded protons (Group 2: B3-F1). The activation energy for B6 has a high degree of error because of the overlap in the peak areas with the F1 end group as discussed above. The activation energy for B2, which is at the N-terminus and not intramolecularly hydrogen bonded, was significantly smaller than that of the hydrogen bonded protons. Intuitively, B1 would also be expected to have this sort of smaller barrier to exchange because this amide proton also lacks the intramolecular hydrogen bond. The intercept is related to the collision frequency (A) in the Arrhenius equation and is a representation of the accessibility of the respective site on the helix. Due to the exponential dependence of A on the intercept ($\ln(A)$), errors were too large to accurately quantify the collision frequencies. The results of this study are summarized on the structure of tBoc-B₆-F1 in Figure 5.



Figure 5. A three-dimensional picture of tBoc-Aib₆-F1 as an ideal 3_{10} -helix (blocking groups are not shown), with hydrogen bonds shown as dashed lines. The fast exchanging amide protons B1 and B2 are not hydrogen-bonded (pale grey), while the slow exchanging B4-F1 amide protons (dark grey) are hydrogen-bonded and not accessible to the solvent. The moderately fast exchanging B3 amide proton (dotted) is hydrogen-bonded, but relatively accessible to the solvent.

DISCUSSION

Previous data shows that the amide protons on residues B1 and B2 (Group 1) are not intramolecularly hydrogen bonded (5, 10, 12). The exchange of these protons is at a fast rate relative to the rest of the amide protons in the helix. This is seen in the large values of the rate constants and by the low activation barrier to exchange. The low barrier to exchange can be explained in terms of lack of the extra stability that would be obtained from hydrogen bonding. That is, there is no increase in bond order for that amide proton. Fast exchange is also expected because these protons are on the end of the helix structure (the N-terminus, Figure 5) and thus they would have more accessibility to the solvent due to the lack of steric hindrance. This is especially true for B1 because there is not another Aib residue on the other side of B1 to cause an increased steric effect.

The amides of B4, B5, B6, and F1 all behaved in a similar manner to each other. Previous data shows that these protons are all intramolecularly hydrogen bonded to a carbonyl within the helix (5, 10, 12). The activation energy for exchange of each of these protons was approximately 80 kJ/mol, which represents the energy needed to form the activated complex (or transition state). This activated complex is the highest energy intermediate in the process of the exchange. Formation of this intermediate requires partial breakage of both the NH bond and the hydrogen bond and thus requires more energy than formation of the activated complex for B1 and B2. The rate constants for the exchange of the amides in the middle part of the chain (B4-B6) are decreased slightly relative to that of F1, which was seen to exchange a little more quickly (Figure 3). The reason for this slight difference in exchange in the F1 group may be attributed to the location of the group on the helix. It is the last group on the C-terminus (the blocking group), and doesn't provide as much steric hindrance as the Aib residues (Figure 5). Amides of B4-B6 all have very low rates of exchange and also have high steric hindrance caused by having dialkylated amides on both sides of their locations. This hindrance probably reduces the accessibility of these protons to the solvent system (reducing the collision frequency) and increases the order of the structure, suggesting a more tightly held together helix in the center than at the ends.

The most interesting proton, B3, exchanged with deuterons quickly, but still is intramolecular hydrogen bonded (5, 10, 12). Further support of the presence of the hydrogen bond is the similar activation energy for B3 to those other residues that are also hydrogen bonded (Group 2). The error in activation energy seen for this proton is minimal because of the amount of data collected and the spectral resolution of the peak, which means that the integration of the peak area is more consistent than that for B6. Therefore, this proton's fast rate of exchange is not explained only in terms of hydrogen bonding stabilization, but rather might be attributed to the collision frequency or entropy effect at that location.

Transition state theory (13) can be used in the thermodynamic interpretation of the activation energy (E_a) and the pre-exponential factor (collision frequency, A) term in the Arrhenius equation (equation 6).

$$k = A e^{-E_a/RT} \quad (6)$$

According to transition state theory:

$$k = Z e^{-\Delta G^\ddagger/RT} \quad (7)$$

where Z is the collision probability and ΔG^\ddagger represents the total free energy change needed to get to the transition state. From the definition of ΔG :

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger \quad (8)$$

combining equation 8 and equation 7 gives:

$$k = Z e^{-\Delta S^\ddagger/R} e^{-\Delta H^\ddagger/RT} \quad (9)$$

Using equation 9 and equation 6, we can interpret the activation energy as an enthalpy change ($e^{-\Delta H^\ddagger/RT} \approx e^{-E_a/RT}$). This energy represents the amount of energy needed for form the activated complex, which is related to the strength of the bonds. The entropy ($e^{-\Delta S^\ddagger/R}$) and the collision probability (Z) terms are the influences on the A term in the Arrhenius equation, meaning that $A \approx Z e^{-\Delta S^\ddagger/R}$. Thus the frequency of collisions and the amount of disorder at the exchange site also influence the exchange rate of the proton.

This suggests that the accessibility of B3 to the solvent might be a factor that also plays a role in the exchange of this proton. This would mean that the entropy and collision frequency are increased at this site in the helix. The greater collision frequency means that the solvent is able to get at the proton more often and interact with more ease. The increase in entropy probably means that the helix might be flexing or slightly moving around a little bit more, but not unfolding because it has been shown to be stable at these temperatures (11).

In conclusion, hydrogen bonding provides greater stability to the helix and an increased barrier to exchange. Another influence on the exchange process is the ability to interact at the site that needs to exchange, and this is attributed to the accessibility. The collision frequency and the effects of entropy of a particular residue within the helix structure determine accessibility. The exchange of a particular proton is thus determined by a combination of both of these above factors.

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