

# Unlocking the Structure and Flexibility in a Four Residue Peptide Using $^1\text{H}$ NMR Spectroscopy

Larry R. Masterson

Faculty Sponsor: Adrienne Loh, Department of Chemistry

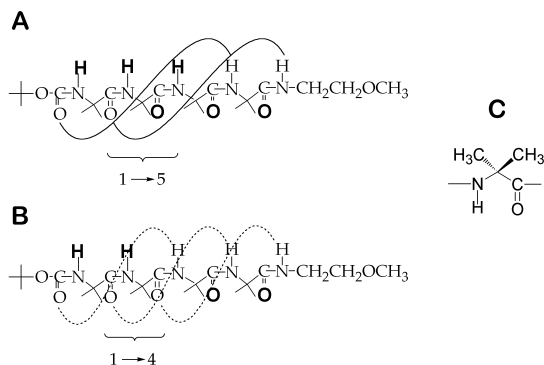
The function of a protein is dictated by its inherent structure and degree of motion. One common substructure found in proteins is the helix. The degree of structure and flexibility of helices is reflected in the relative strengths of the hydrogen bonds within the helix. As models of helices, a short  $3_{10}$ -helical polymer of the dialkylated amino acid Aib ( $\alpha$ -aminoisobutyric acid) was studied. The accessibility of the amide groups of  $\text{tBoc-(Aib)}_4\text{-NHCH}_2\text{CH}_2\text{OCH}_3$  to a deuterated solvent (methanol) was studied by monitoring the decay of the amide proton NMR signals over time due to the exchange of the amide protons with the solvent deuterons at several temperatures. The rate of peak area decay is related to the frequency of solvent interaction of a particular amide proton in the structure, and thus to its hydrogen bond strength. An analysis of the results using pseudo-first order kinetics reveals that the two N-terminal amides exchange quickly at all temperatures, while the two C-terminal amides exchange very slowly at all temperatures. The central amide proton also exchanges very slowly at low temperatures, but at a more moderate rate at higher temperatures. This behavior is reflected to some extent in the activation barriers to exchange, which are low for the N-terminal amides, moderate for the central amide, and high for the C-terminal amide. These results indicate that the helical structure of  $\text{tBoc-(Aib)}_4\text{-NHCH}_2\text{CH}_2\text{OCH}_3$  is more flexible at the N-terminus, and that it is destabilized as temperature is increased.

## INTRODUCTION

Proteins are organic compounds that have structural, enzymatic, and regulatory roles in biological systems and are composed of chains of amino acids (1, 2). The information gained from unlocking the structural properties of proteins is useful in understanding what governs many biological functions. While the diverse array of protein structures makes this study quite complex, short sequences of amino acids can be used as models for structures in proteins. One structure found in peptides and proteins is the helix, which can be thought of as a single stranded spiral or coil. The purpose of this research is to obtain some information about the structure and dynamics of a four residue helical peptide by studying the kinetics of the exchange of hydrogen for deuterium at the amide protons using  $^1\text{H}$  Nuclear Magnetic Resonance (NMR) spectroscopy.

Helical structures are stabilized by a H-bond between the carbonyl group on one amino acid and the amide group on a different amino acid (3). In proteins, helices typically have H-bonding across four amino acids, a pattern commonly recognized as characteristic of the

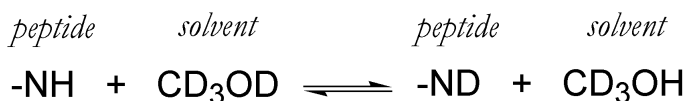
$\alpha$ -helix (Figure 1A). Most peptides made of common amino acids do not form helices, however, peptides as short as three residues in length that are made of the unusual amino acid,  $\alpha$ -aminoisobutyric acid (Aib) (Figure 1C) do form helices (4). These peptides tend to form helices that have H-bonding across three amino acids, a pattern characteristic of the  $3_{10}$ -helix (Figure 1B).



**Figure 1:** Helical hydrogen bonding patterns in a four residue peptide. **A)** The  $\alpha$ -helix has an  $i \rightarrow i + 4$  intramolecular H-bonding pattern. The notation of  $i \rightarrow i + 4$  refers to the H-bonding of the carbonyl on residue  $i$  to the amide hydrogen of residue  $i + 4$ . **B)** The  $3_{10}$ -helix has an  $i \rightarrow i + 3$  intramolecular H-bonding pattern. This pattern causes the two N-terminal amide protons to be non-hydrogen bonded. **C)** The Aib ( $\alpha$ -aminoisobutyric acid, B) residue. This unusual amino acid is dialkylated at the  $\alpha$ -carbon.

The  $3_{10}$ -helix is interesting because it is rare, although it is found in some proteins (3). Research has suggested that this helix may be an intermediate in the folding pathway of the more abundant  $\alpha$ -helix (5,6). It has also been proposed that the  $3_{10}$ -helix is involved in the binding sites of some enzymes where biochemical processes take place (7). Additionally, pursuit in the study of Aib proves beneficial in view of the fact that it accounts for 30-50% of the amino acids in channel-forming cell membrane proteins in bacteria (8).

The peptide tBoc-Aib<sub>4</sub>-NHCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub> (shown in Figure 1B) has been shown to adopt a  $3_{10}$ -helical conformation in a variety of solvents and in the solid state (4,9). This investigation involves the exchange of amide hydrogens on this peptide for deuterium from the solvent (Figure 2). The accessibility of each amide to the solvent and thus the relative strength of each hydrogen bond can be monitored at each particular location on the helix using <sup>1</sup>H NMR spectroscopy. The exchange reactions are performed at different temperatures, allowing a more thorough determination of H-bond strength and helix flexibility by determining the activation energy for the exchange reaction.



**Figure 2:** Hydrogen (H)-deuterium (D) exchange reaction. In this reaction, the proton on the amide group from the peptide (-NH) exchanges with a deuteron from the solvent ( $\text{CD}_3\text{OD}$ ). Deuterons are not detected by the  $^1\text{H}$  NMR instrument. Because of the excess  $\text{CD}_3\text{OD}$  used, the equilibrium lies very far to the right.

## METHODS

$^1\text{H}$  NMR spectra were recorded at different time intervals on a Bruker AC3000 300 MHz spectrometer using a 4mM solution of tBoc-Aib<sub>4</sub>-NHCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub> in  $\text{CD}_3\text{OD}$ . Time was started precisely when  $\text{CD}_3\text{OD}$  was introduced to the sample. The tip angle for each pulse was  $52^\circ$  and the number of scans was 16. Reaction temperatures were 275 K, 286 K, 303 K, 309 K, and 315 K.

The spectra were processed using the software program WinNUTS (Acorn NMR). After Fourier transformation and phasing, all spectra were corrected using polynomial baseline corrections. The integrated intensity of each amide hydrogen signal was calculated relative to the methyl protons of Aib and tBoc, which do not exchange with  $\text{CD}_3\text{OD}$ . To ensure consistency with each spectrum in a given experiment, all subsequent spectra at different time intervals but at the same temperature were processed with the same baseline corrections and integral phasing.

The rate law for the reaction in Figure 2 is given by equation (1):

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

Since the reaction is performed under conditions where the solvent is flooded, the concentration of  $\text{CD}_3\text{OD}$  can be treated as a constant. The pseudo first order rate law is therefore given by:

$$\text{Rate} = k'[\text{NH}] \quad (2)$$

where the pseudo first order rate constant is:

$$k' = k[\text{CD}_3\text{OD}] \quad (3)$$

Values for  $k'$  were obtained using the integrated form of the rate law equation:

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

where  $I$  is the relative peak area of an amide proton,  $I_0$  is the relative peak area at  $t=0$ ,  $k'$  is the pseudo first order rate constant, and  $t$  is the time at which the relative peak area is measured.

Values for the activation energy ( $E_a$ ) were determined from a linear plot of the Arrhenius equation,

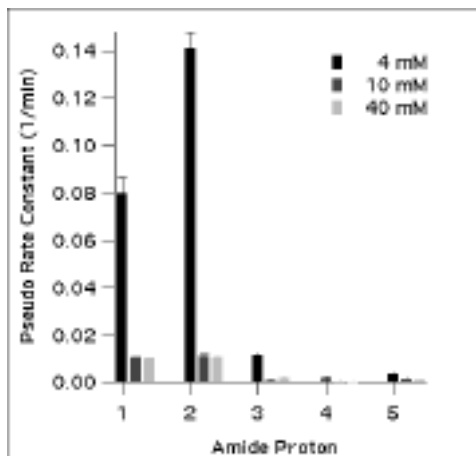
$$\ln k' = \ln A - \frac{E_a}{RT} \quad (5)$$

where  $A$  is the collision frequency,  $E_a$  is the activation energy,  $R$  is the ideal gas constant, and  $T$  is temperature in K. Values for the collision frequency were not determined because of the high error that is associated from this small range of temperatures. All fits were performed using linear least squares analysis and errors were reported as standard deviations.

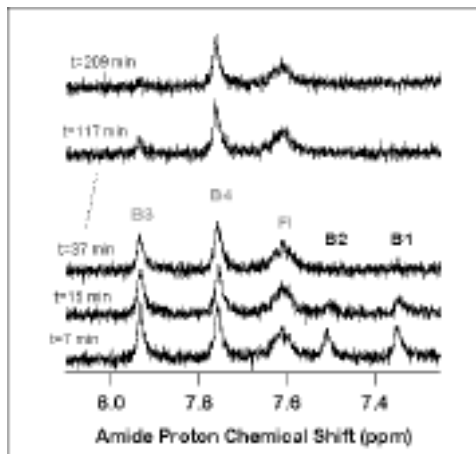
The effect of solution concentration on the exchange behavior was studied at 309 K and concentrations of 4 mM, 10 mM, and 40 mM. All acquisition parameters and processing procedures were the same as described above.

## RESULTS

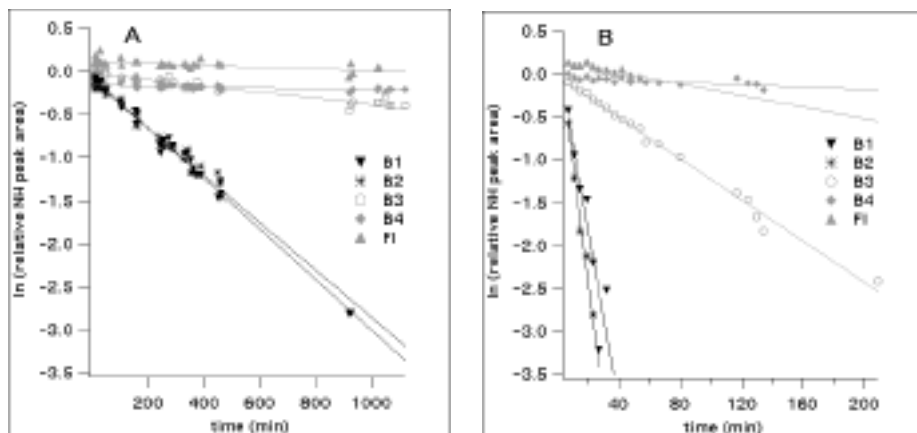
Aib-containing peptides have been shown to aggregate, or intermolecularly hydrogen-bond, in  $CDCl_3$  solutions at concentrations greater than 10 mM (4), while aggregation is negligible in DMSO- $d_6$  at similar concentrations (10). The first set of experiments was therefore to determine the concentration at which the kinetics studies should be performed. At 309 K, it was found that the rate constants for amide hydrogens undergoing the exchange reaction were similar at high concentrations (40 mM and 10 mM). However, when the concentration was decreased to 4 mM, the pseudo rate constants increased drastically for amide hydrogens B1 and B2 (Figure 3). This can be explained in terms of peptide aggregation, which decreases the accessibility of the solvent to the amide hydrogens in the helix. When these hydrogens are no longer intermolecularly hydrogen bonded to the carbonyl oxygen of another peptide, they become more accessible to the deuterated solvent and thus exchange more quickly. Interestingly, the rate constants for B4 and F1 (the amide proton of the blocking group



**Figure 3:** Values for the pseudo-rate constants for the exchange of amide protons in tBoc-(Aib) $_4$ -NHCH $_2$ CH $_2$ OCH $_3$  for solvent deuterons in CD $_3$ OD at 309 K for three concentrations. Amide 5 belongs to the C-terminal blocking group, NHCH $_2$ CH $_2$ OCH $_3$  ("F1").



**Figure 4:** The  $^1H$  NMR spectra of the amide region of tBoc-Aib $_4$ -NHCH $_2$ CH $_2$ OCH $_3$  as a function of time at 309 K. These spectra depict the decay in the concentration of amide hydrogens as they are exchanged for deuterons from the solvent. The amides with gray lettering are intramolecularly bonded, while those with black lettering are not.



**Figure 5:** The pseudo first order plot of the exchange reaction at **A)** 276 K and **B)** 309 K. The linear relationship shown here is justification of the pseudo first order kinetics used to model this system. The pseudo first order rate constants were obtained from the slopes of these lines.

NHCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>) are much smaller at the lower concentration than at higher concentrations. This may be a result of helix distortion during aggregation since it is the carbonyl oxygens of B4 and F1 that form hydrogen bonds with the amide protons of B1 and B2 (on a different peptide molecule) during aggregation. Based on these results, all subsequent experiments were conducted at a concentration of 4 mM.

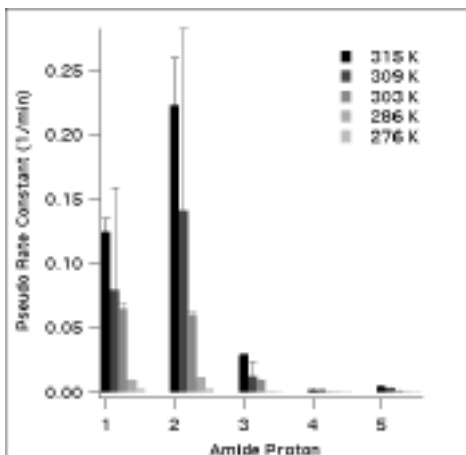
Sample spectra from an exchange experiment performed at 4 mM and temperature of 276 and 309 K are shown in Figure 4. It is evident from this data that there are two groups of amide hydrogens in the tBoc-Aib<sub>4</sub>-NHCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub> peptide. Group 1, which includes amide hydrogens B1 and B2, exchanges relatively fast (no peaks are evident after 37 minutes). Group 2, which includes amide hydrogens B3, B4, and F1, is relatively slow to the exchange reaction. This difference in behavior between the two groups of amide hydrogens concurs with the intramolecular amide hydrogen-bonding pattern (described in Figure 1B), in which the first two amide hydrogens (B1 and B2) in the peptide are non-hydrogen-bonded, while the other amide hydrogens (B3, B4, and F1) are hydrogen bonded. However, the amide hydrogen B3 does seem to display a somewhat faster exchange relative to the other amide hydrogens in this group.

For each temperature, pseudo first order rate constants were determined for each amide hydrogen using equation 4 (Figure 5). The slope of the line is proportional to the pseudo first order rate constant for that amide hydrogen, so that steep slopes are indicative of larger rate constants, while more gradual slopes are indicative of smaller rate constants. Equation 4 shows that the y-intercept is related to the initial amide hydrogen concentration in solution. The intercepts did not equal zero (the initial relative intensity was not 1) since the amide deuterons may have not been completely exchanged back to hydrogens from previous experiments. This does not affect the experimental results since only the change in the concentration is significant.

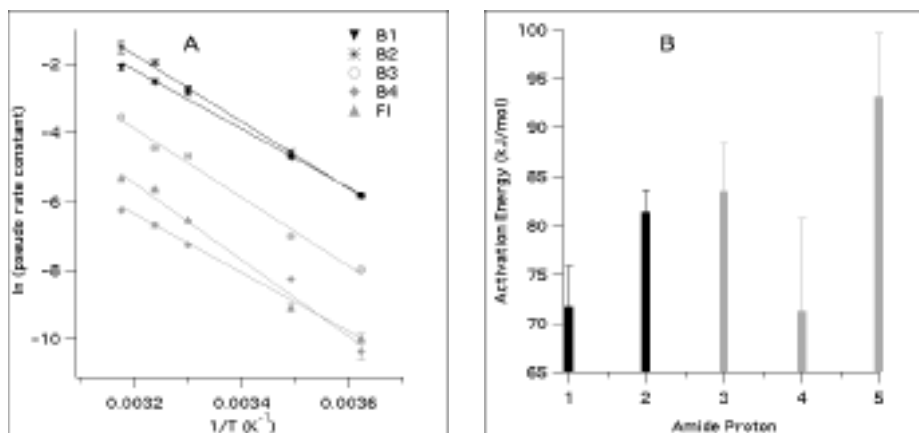
A summary of the rate constants for the temperatures that were studied is given in Figure 6. The differing behaviors of the two groups of amide hydrogens that were mentioned above are again apparent in this data. Group 1 has relatively large rate constants, while Group 2 consistently has relatively small rate constants. Within Group 2, however, amide hydrogen B3 has a variable behavior, with small rate constants at lower temperatures, but somewhat larger pseudo rate constant at higher temperatures.

The activation energies for the exchange reaction for each amide were found by performing a linear least squares fit of the rate constant data to equation 5 (Figure 7A). The slope of the fit line is indicative of the activation energy for the exchange reaction at that location (Figure 7B). The same two groups of amide hydrogens are evident from this data also. Group 1 amides (B1 and B2) have small activation energies, and are therefore more easily exchanged for deuterium. Group 2 amides (B3, B4, and F1) have larger activation energies and are therefore more difficult to exchange for deuterium. The intermediate behavior of B3 is less evident from these data, which may suggest another mechanism is responsible for the intermediate behavior of B3 at elevated temperatures.

The activation energy for the exchange reaction at B4 has a very high degree of error. It is believed that the actual value of the activation energy for the exchange reaction at B4 is closer to the maximum value with the error incorporated. There are two possible reasons that would explain this high error. The first, and most probable reason, is that it is a result of a very slow exchange that occurs at this location with the temperatures that these experiments had been done. Since the exchange was so minimal, even at long durations of over 1100 minutes, the slopes of the pseudo first order plots were very gradual. Therefore, less reliability would be obtained in the measurement of the slopes for the pseudo rate constants. A second explanation for this high error may be that the collision factor,  $A$ , (see equation 5) is not considered. Because of its high degree of disorder, the C-terminal blocking group, F1, may be interfering with the accessibility of the solvent to the amide hydrogen at this location. The collision factor can be obtained by extrapolating the Arrhenius plot back to the y-intercept (which is at very high, or infinite temperature on the x-axis at this point). Unfortunately, a reliable estimate of the collision factor cannot be obtained from the experimental data that is available due to the small temperature range studied and the exponential dependence of the error in the fit on the intercept.



**Figure 6:** Pseudo rate constants for the exchange of amide hydrogens in  $t\text{Boc}-(\text{Aib})_4\text{-NHCH}_2\text{CH}_2\text{OCH}_3$  at five different temperatures. It is apparent that there are two groups of amide hydrogens from this data. The first group has very large pseudo rate constants, while the second group has relatively small pseudo rate constants.



**Figure 7:** **A)** The Arrhenius plot for the exchange reactions performed on tBoc-Aib<sub>4</sub>-NHCH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>. **B)** The activation energies for the exchange reactions for each amide hydrogen.

## DISCUSSION

The exchange of an amide hydrogen for a solvent deuteron ultimately occurs through interaction of the amide with the solvent. Amides that are able to interact with the solvent frequently will exchange more quickly than those that are not. The accessibility of an amide to the solvent is dictated in part by the flexibility of the helix at that site. This in turn is influenced by the strength of the intrahelical hydrogen bond to that amide, since the  $3_{10}$ -helical structure is stabilized by these interactions. Therefore, amides in less flexible regions of the helix are expected to have stronger hydrogen bonds, and thus higher activation energies for exchange than those amides where no hydrogen bonding occurs.

The large rate constants and low activation energies that are associated with the exchange reaction for the amide hydrogens B1 and B2 are in agreement with the reported  $3_{10}$ -helical structure of this peptide. Since these amides are not hydrogen bonded, they are very accessible to the solvent, and thus undergo exchange quickly. The fact that these amides exchange with low activation barriers most likely indicates that the helix is flexible in this region. Indeed, low activation barriers to exchange have been observed in an eight residue Aib-rich peptide for hydrogen-bonded amides in a region of increased flexibility, indicating that helix flexibility is correlated with the magnitude of the activation barrier (11). The activation barrier may also be low because additional energy does not need to be used to break a hydrogen bond in order to reach the transition state for the exchange reaction. In this scenario, the amides are especially accessible to the solvent because they are not “protected” by hydrogen bonds. This sort of information may also be contained in the frequency factor, A. Values of A are notoriously difficult to obtain with any accuracy due to long range extrapolation required to reach the intercept of the Arrhenius plot and the exponential dependence of the error in A on the intercept. In all probability, both factors are significant in determining the activation barrier for these non-hydrogen bonded amides.

The lower exchange rate constants and relatively higher activation energies that are required to exchange the amide hydrogens B3, B4, and F1, are also in agreement with the

reported  $3_{10}$ -helical structure of this peptide. These amide hydrogens are hydrogen bonded and are less accessible to the solvent than B1 or B2, as indicated by the dramatically lower exchange rate constants for B3, B4, and F1. These trends were also apparent in similar experiments on a six residue Aib peptide (12). The decreased accessibility is presumably due to decreased flexibility of the helical structure at this area of the molecule, due in part to stronger hydrogen bonds (and thus larger activation barriers to exchange). However, this decreased flexibility is also a result of the accumulated steric hindrance in the Aib sidechains due to the dialkylation at the  $\alpha$ -carbon. Previous results have indicated that  $3_{10}$ -helices composed of Aib become more regular in structure as the length of the helix increases (4, 9). Unfortunately, comparison of the results reported here with those from the Aib<sub>6</sub> peptide (12) are difficult to make as the two studies were carried out at different concentrations.

Inferences about the relative strengths of the hydrogen bonds to B3, B4 and F1 are further complicated by the high degree of error that is associated with activation energy for the exchange reaction at B4. A more reliable value for the activation energy can be obtained by elevating the temperature to increase the rate of the exchange reaction for this very slowly exchanging amide. This will create a steeper slope for the determination of the pseudo rate constant. Unfortunately, deuterated methanol will reach its boiling point at about 330 K, so only a few more elevated temperature data points are possible. A different solvent with a higher boiling point such as deuterated ethanol may be explored in future experiments. Overall, a length dependent study of homo-Aib peptides is being pursued in order to help discover how the length of these peptides will affect their helical dynamics. Thus, conditions such as the choice of solvent will be kept as a controllable variable for this series.

An interesting observation was that although B3 seems to be part of the second, more slowly exchanging group of amide hydrogens, it appears that B3 begins to act more similarly to the first group at higher temperatures (309 K and above, see Figure 5). This may be a result of the hydrogen bond being broken or weakened as the temperature is increased. Due to the steric constraints imparted by the Aib sidechains, the helix cannot truly unfold (13). Thus a weakened or broken hydrogen bond does not mean that the helix is unfolding, but rather it is less stabilized at these elevated temperatures. A two-dimensional ROESY  $^1\text{H}$  NMR study of this peptide that was performed in DMSO- $d_6$  had shown that the peptide is  $3_{10}$ -helical at room temperature (9). Similar experiments in methanol at a variety of temperatures might reveal the extent of helix destabilization. Alternatively, it has been shown that the chemical shift of non-hydrogen bonded amide protons is very sensitive to the effects of changing the solvent (4,9,11). Experiments in which these effects are measured at a variety of temperatures may reveal more information on the hydrogen bonding status of B3.

## ACKNOWLEDGMENTS

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