Assessing the Effect of a Double Histidine Motif on Helix Tilt in Membrane Environments

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Abstract

Proteins are very important to biological systems and therefore their structure area of great interest in biological research. This research aims to see if a previously used dHis motif can be used in membrane environments, which have previously not been able to be studied. This research is considered ongoing and incomplete as of now. Two peptide sequences of interest have been successfully synthesized, although they are currently impure.

Introduction

Proteins are very important; they are within every living organism and they are responsible for the many regulatory tasks that living organisms need to happen in order to survive.[1] Proteins consist of a chain of amino acids that are varying length.[1] These amino acids then bond together via peptide bonds to form the primary structure of the proteins.[1] The chain then folds on its self to form α-helices or β-sheets, which are the secondary structure of the proteins.[1] The varying secondary structures within one amino acid chain is the tertiary structure.[1] The way the protein folds into its secondary and tertiary structure is vital for the protein to function.[1] If the protein folds incorrectly in any way, the protein will be unable to function which could potentially cause devastating effects within a living organism including genetic disorders.[1] Since protein structure and conformation is so important to its function, the determination of this structure is very important in biochemical research.

One method that has been used in protein structure determination is Electron Spin Resonance (ESR).[2] ESR is similar to Nuclear Magnetic Resonance, except that it uses the spin of an unpaired electron to help determine molecular structure.[2] One specific type of ESR is Double Electron Electron Resonance (DEER); this type of ESR not only provides structural data but allows scientists to determine the distance between two unpaired electrons.[3] ESR and DEER are often used in combination with spin labels.[3]

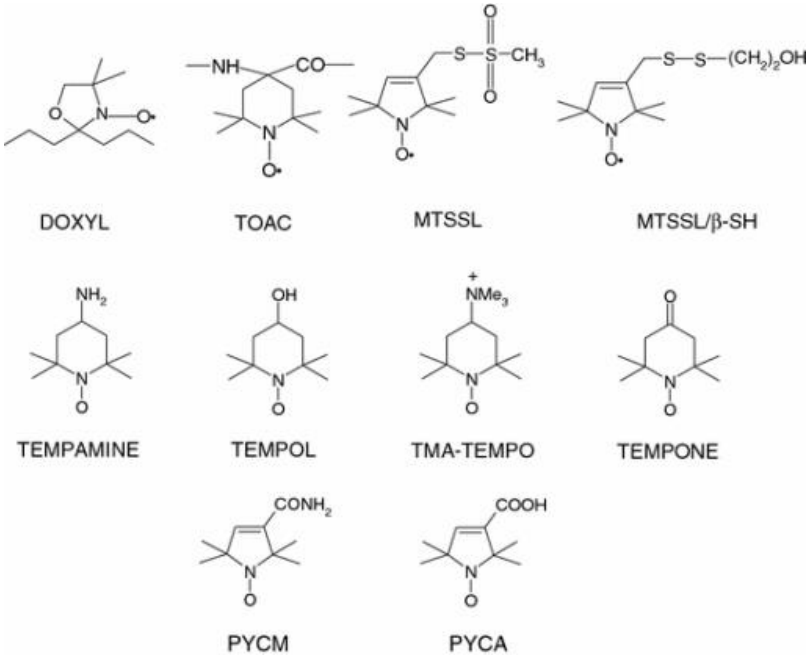

Figure 1: Common spin labels used in ESR (National Institute of Medicine)

Spin labels are important tools; some common spin labels are shown in Figure 1. Many spin labels use N-O bonds and utilize a free radical that can be found on the oxygen.[5] Paramagnetic metals can also be used as spin labels in ESR.[2] Traditionally, spin labels have been attached via the side chain of the amino acids.[6] However, because of the flexibility of these side chains, the distance measurements can be difficult to interpret and obtain the actual distances.[6] Another problem with several spin labels, is that they have an environmental bias.[7] They prefer to be in a hydrophobic area of the protein, which means that the membrane area of the peptide, which is the environment that a large number of proteins exists, is unable to be studied with current methods.[7] 2,2,6,6-Tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid (TOAC), which can be seen in Figure 1, helped to alleviate the flexibility problem of other spin labels.[6] TOAC can be used as a spin label in peptides and is attached to the peptides via a peptide bond.[6] This means TOAC is part of the amino acid sequence and that it does not have the flexibility of other spin labels.[6] One problem with TOAC, however, is that it cannot be easily used in proteins.[6] It is an artificial amino acid which means biological systems do not produce it naturally and would be difficult to incorporate into protein production.[6] Therefore, a new spin label is needed to readily obtain protein structure data in membrane environments and that can be readily produced in natural environments.

One spin label that shows potential in being used within membrane environments is a double-histidine (dHis) motif.[8] This motif has already been successfully used as a spin label in proteins as the histidines have the ability to bind to paramagnetic metals.[8] Histidine has been known to alter the alignment of certain peptides in regard to what is normal for the membrane, which could be a problem when using d-His as a spin label.[9] Therefore, the first step in gauging if dHis is the ideal candidate for a spin label to determine distances in membrane systems which would help in the study of protein structures, because, again, a large number of proteins exist in this environment.

The goal of this research is to determine the effect of the dHis motif on the tilt of the helix relative to the lipid bilayer. In order to determine the affect of the dHIs, a WALP peptide will be used.[10] A WALP peptide is a common α-helix peptide that consists of tryptophan (W), alanine (A), leucine (L) and easily embeds itself into the lipid bilayer.[10] This type of peptide has been used in the past to investigate the alignment of peptides in the lipid bilayer.[10] This will be done using five peptide sequences of 23 amino acids

each. The amino acid sequences that will be synthesized are shown in Figure 2 and will be synthesized using solid phase peptide synthesis.

Peptide	Sequence
1	WWWLALALALALALALALWWW
2	WWWLALALALALALALHLWWH
3	WToWLALALALALALALHLWWH
4	WWWLALALAHALAHALALALWWW
5	WToWLALALAHALAHALALALWWW

Figure 2: The 5 peptides that will be synthesized and analyzed within the lipid bilayer

The amino acid sequences are twenty-three amino acids long as this length matches the thickness of the 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayer. This is a lipid bilayer that is used commonly for this type of experiment and can be found in living systems.[11] This type of peptide will fold when exposed to the lipid bilayer. The tryptophans, which are more polar will be closer the outside of the bilayer in the more hydrophilic environment. The leucines and alanines, which are more nonpolar, will be inside the bilayer in the hydrophobic region. The dHis motif will be implemented in different regions. In peptides 2 and 3, the histidines will be in the more hydrophilic region. In peptides 4 and 5, the histidines are placed in the more hydrophobic region of the peptide sequence. The dHis motif is going to be placed in both regions in order to determine if there is a bias, and it is better in one environment over another, which is a common problem with spin labels. Peptide 1 will serve as the base peptide in order to see how it tilts in the bilayer before any histidines are incorporated. The TOAC (shortened to To in Figure 2) is incorporated in so a previously determined method of ESR can be used in order to determine the affect the histidines have on the tilt of the helix.

Materials and Methods

Solid phase peptide synthesis was used in order to synthesis the needed peptides as shown in Figure 2. In the first attempt at making peptide 1 (shown above), an unloaded 2-chloryltrityl chloride resin was used. The resin was allowed to swell in of dichloromethane (DCM) for fifteen minutes and then filtered. Then the L-tryptophan was loaded onto the resin by mixing the tryptophan (1 equivalent), 2 equivalents of N,N-Diisopropylamine (DIPEA), and DCM (0.05 M solution). This solution was allowed to rotate for one hour. After rotating, the mixture was washed five times with DCM and five time with a 50% methanol (MeOH) in DCM and then left to dry overnight. The loading of the resin was then determined. This was done by swelling three vials (10 mg each) of the loaded resin with dimethylformamide (DMF) for twenty minutes. Piperidine (.2 mL) was then added to each vial. A blank was prepared using a 0.01 M solution of piperidine in DMF. The absorbance of each vial was found in a Ultraviolet-Visible Spectroscopy (UV-VIS) and the loading of each was calculated. The loading was calculated using equation 1.

$$loading = \frac{101 * absorbance}{7.8 * mass\ in\ vial} \qquad [1]$$

After the loading was determined, the first coupling reaction was performed. The resin was swelled with DMF for fifteen minutes. After swelling, a solution consisting of tryptophan (5 equivalents), the coupling agent 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one, DEPBT, (5 equivalents), DIPEA (10 equivalents), and DMF (0.5 mol % of amino acid in DMF), was mixed together and then added to the resin. This mixture rotated for four hours and then washed with DMF until the filtered liquid was no longer yellow in color. The newly added tryptophan was then Fmoc deprotected using 20% piperidine in DMF by adding the piperidine/DMF solution to the resin and rotating for three minutes and then filtering. This was repeated two more times. The loaded resin was then washed with DMF ten times. Each following coupling reaction was done following the same process and solution amounts. The were only rotated for two hours each. After the last coupling reaction was performed and the solution deprotected, the resin was cleaved from the peptide. The resin was first washed with MeOH and then washed with DCM. The resin was then dried, and afterword swelled with DCM. The DCM was filtered from the resin and the cleaving solution was added. The cleaving solution consisted of 20% hexafluroisoproponal (HFIP) and 80% DCM. The resin and cleaving solution was rotated for one hour and filtered into a tared flask; this process was repeated once more on the previously cleaved resin. The HFIP/DCM solution was evaporated off the cleaved peptide using a rotary

evaporator and then left to dry using a high vacuum. Thin layer chromatography (TLC) was used to check for purity. The solvent system used was 10% MeOH in chloroform. The second attempt at loading the resin followed the same procedure as above. After the initial loading, no further coupling reactions were performed on this loaded resin.

The next attempt at making peptide 1 and the only attempt at making peptide 2, used 2-chlorytrityl chloride resin pre-loaded with tryptophan, so the loading was already known. All coupling reactions and the cleaving of this resin follow the same procedure as outlined above. For the second try at peptide 1 and for peptide 2, 20% MeOH in chloroform was used as the solvent system when TLC was performed.

An attempt at synthesizing 2,2,6,6-Tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid (TOAC) used the following procedure. The first step of the synthesis of TOAC is converting 2,2,6,6-tetramethyl-4-oxo-piperidone into a nitroxide and used the methods outline by Smythe.[12] The first try at this was done by dissolving 1 equivalent of the piperidone into enough MeOH to make a 0.09 M solution. Then Na_2WO_4 x $2H_2O$ (.17 equivalents) was added to this solution. 6 equivalents of 30% hydrogen peroxide was added dropwise. After five hours of stirring, six more equivalents of hydrogen peroxide were added. This was repeated after 24 hours. Also, after 24 hours of stirring, 0.085 equivalents of Na_2WO_4 x $2H_2O$ was added to the solution. The progress of this reaction was followed using TLC; the solvent system used was 70% ethyl acetate in hexanes. A workup was performed and then column chromatography was performed. The solvent systems used were 25% ethyl acetate in hexanes, 50% ethyl acetate in hexanes, and 60% ethyl acetate in hexanes.

The next attempt at converting the piperidone into nitroxide by combing the methods outlined by Schulte[13] and Smythe[12]; the methods outline by Smythe were also used for all continuing reactions for the synthesis of TOAC. 1 equivalent of the piperidone, .17 equivalents of Na_2WO_4 x $2H_2O$, and six equivalents of hydrogen peroxide were mixed together and stirred on ice for four days. The aqueous layer of the solution was saturated with K_2CO_3 and washed with ethyl acetate (3 x 20mL). No further purification of the product was necessary after checking for purity via TLC.

The next step of the TOAC synthesis is converting the formed nitroxide into a hydantoin. This was done by mixing together the nitroxide (1 equiv), ammonium carbonate (12 equivs), 1/1 ethanol and water solution (0.4 M), and 2.54 equivalents of sodium cyanide. The mixture was allowed to reflux at 60°C until

the reaction was done, as monitored on TLC using 50% ethyl acetate in hexanes. The product was extracted using ethyl acetate (3 x 15mL) and dried with Na_2SO_4.

The final step in the synthesis of TOAC is forming TOAC from the previously formed hydantoin. This was done by mixing together the hydantoin (1 equiv), BaOH x $8H_2O$ (7 equivs), and water (enough to form a 9 M solution). The resulting mixture was refluxed for four days. The resulting solid was gravity filtered and washed with water. It was then dissolved in 1 M sulfuric acid and loaded onto a dowex column. 0.5 M NH_4OH was used to elute the product. It was then dried using low heat and air pressure. The Fmoc protection step of TOAC was not performed in this experiment.

Results

Sample	Mass (mg)	A	Loading
1	10	.158	.205
2	10.5	.225	.277
3	10.6	.229	.280

Table 1: Absorbances and loading found for the first attempt at loading resin for making peptide 1. Average loading was .250

Sample	Mass (mg)	A	Loading
1	10.1	.620	.795
2	10.2	.423	.537
3	10.4	.502	.625

Table 2: Absorbances and loading found for the second attempt at loading resin to make one of the intended peptide sequences. This attempt was not continued. Average loading was .652.

Figure 3: TLC of the first finished attempt at peptide 1

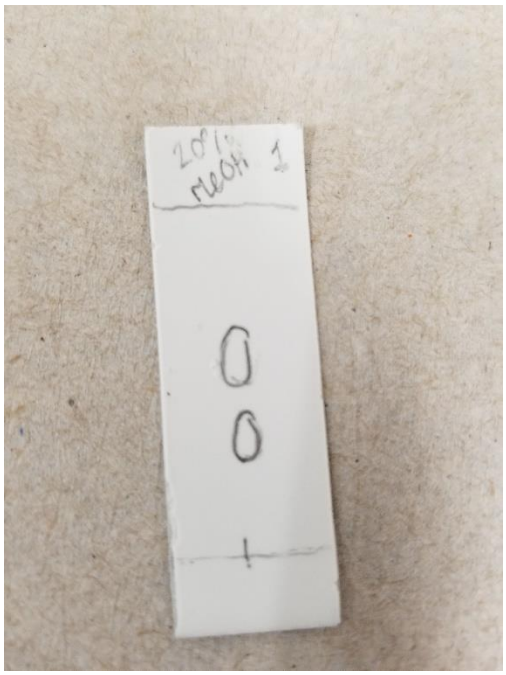

Figure 4: TLC of the second finished attempt at peptide 1 (pre-loaded resin was used)

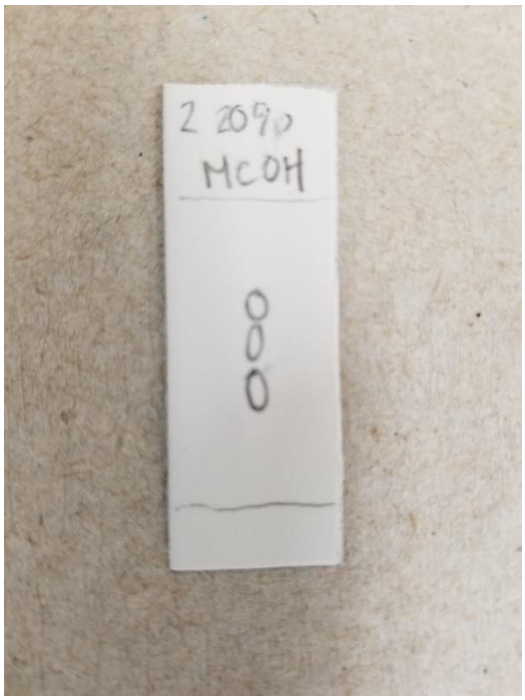

Figure 5: TLC plate of finished peptide 2 made with pre-loaded resin.

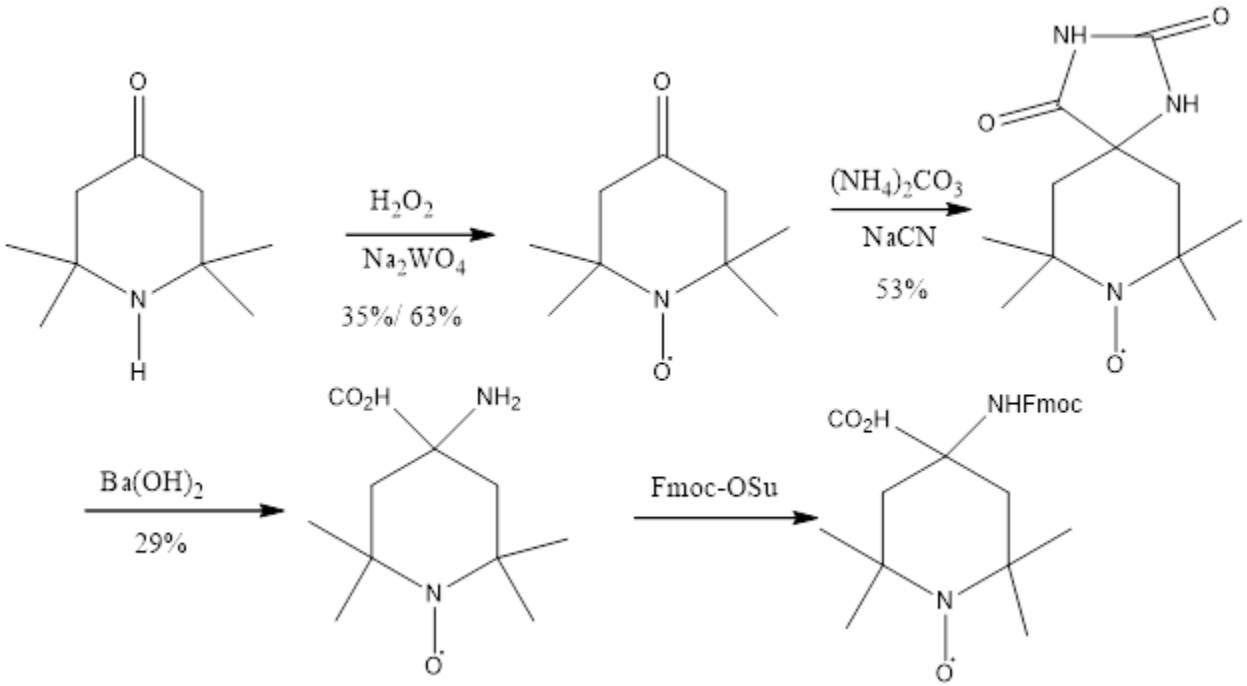

Figure 6: Synthesis of TOAC with the relative percent yields for each attempt of each step of the synthesis

Discussion

The goal of this research was to determine what the effect the d-His motif has on the orientation of the peptide in a lipid bilayer. This research was started with the procedures outlined here, but to ultimately be able to determine if the d-His can be used in a lipid bilayer, there needs to be continuing research done on this subject. Currently with this research, two peptides (peptides 1 and 2) have successfully been synthesized. They are not pure, however.

Figure 3 as shown in the results shows purity of the peptide made using the unloaded resin. As made obvious by the multiple spots, it is impure. In the next attempt to make a peptide, the loading was found to be very different. The first attempt had an average loading of 0.250 whereas the second attempt to load the resin had an average loading of 0.652. That led us to believe that the original loading found was incorrect. In order to prevent this problem from potentially occurring again, we did not continue with the second attempt at loading the resin; we instead synthesized the peptides using resin that was pre-loaded with tryptophan. Peptide 1 made using pre-loaded resin was less impure, but still not pure. This can be seen by comparing Figure 4 with Figure 3. There are only two spots on the TLC plate in Figure 4, meaning there are fewer impurities. Figure 5 shows the TLC plate used to asses the purity of synthesized peptide 2. It is also impure, as it also has multiple spots.

While it has not been performed in this research, size exclusion chromatography is likely a good method for purify these peptides. The reason size exclusion is a likely candidate, is due to the fact that the impurities in the peptides are likely just shorter peptide chains with deletions in various locations. The shorter chains will likely have all the same types of amino acids, just in differing amounts. Because of this, they will have the same polarity and therefore cannot be purified using normal column chromatography. High Performance Liquid Chromatography (HPLC) purification is not a viable option because the technology needed to perform this type of purification is not currently readily available at Hanover College. So, if this research is continued, size exclusion chromatography is a viable method to try.

An attempt at synthesizing TOAC was made in this research, as it will be used in further research as a spin label for the peptides. The synthesis had low to moderate yields (as seen in Figure 6), but also was not able to be characterized at Hanover. After the nitroxide was performed, Nuclear Magnetic Resonance (NMR) was performed, but the results were inconclusive. The spectra gave no data and it was

concluded that the NMR at Hanover likely was not able to give structural data because of the free radical electron present in the nitroxide product. An Infrared (IR) spectra was also not able to be obtained past forming the nitroxide. This was possible due to low amount of product formed. The last step, which was the Fmoc protection step, was not performed and ultimately the attempt at synthesizing TOAC at Hanover, was stopped. Instead, we bought already synthesized and protected TOAC.

In conclusion, we were able to successfully synthesize the peptides, a successful purification method will be the next step. While there is not any data on how d-His affects the orientation of the peptide in the lipid bilayer, this goal can be continually worked on.

Moving Forward

The next step of this research is to find a successful purification method; this may likely be size exclusion chromatography as we already have resin that can be used for the size of peptide that was synthesized. After all the peptides have been successfully synthesized and purified, the effect of the histidines on the tilt of the peptide within the lipid bilayer can be determined using ESR.

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