Chapter 4: Future Directions

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4.1 Summary of Dissertation

In the previous chapters, I detailed the research in the Senes lab that has focused on understanding driving forces in MP folding. My research focused on improving our understanding of the impact that van der Waals packing has on facilitating MP folding and association. We studied a subset of van der Waals packing known as sidechain packing and found that sidechain packing is a weak driving force when it is the sole force used to design the association between TMHs.

In my recently submitted 2024 paper (Chapter 2), I studied the effect of sidechain packing on thousands of MP dimers. Through computational design and mutagenesis of key interacting residues, we found that proteins designed to associate with solely sidechain packing dimerized less than proteins designed with both sidechain packing and interhelical hydrogen bonding (GASright). Additionally, our energetics correlate much better to the GASright region, suggesting that our computational model is not well tuned to predict the association of weak MP dimers. In Chapter 3, I detailed many of the computational methods that I developed to design dimers and the rationale for decisions made during the design procedure. Explaining these methods in detail allows for students in our lab and others to utilize and/or co-opt my methods for future research.

My research has shown that sidechain packing is an essential force for MP folding and association, despite being a weak driving force. In this chapter, I expand on potential avenues for future research where this knowledge can be utilized. I first discuss additional experiments that were not included in my publication, where I studied the effects of mutating out all potential hydrogen bonding residues used in the design process on a subset of designs. These results confirm that we designed multiple dimers that associate by sidechain packing in the absence of any potential hydrogen bonding. I then suggest future experiments to enhance our understanding of sidechain packing in the presence of other forces. I detail my ideas for potential improvements to my protein design algorithm, including the potential for designing heterodimer sequences, converting sequence entropy into a pairwise term, and improving our energetics using machine learning. Finally, I suggest improvements for our high-throughput sort-seq method, with attempts to further characterize sequences by their expression level in cells.

4.2 Hydrogen bond mutations

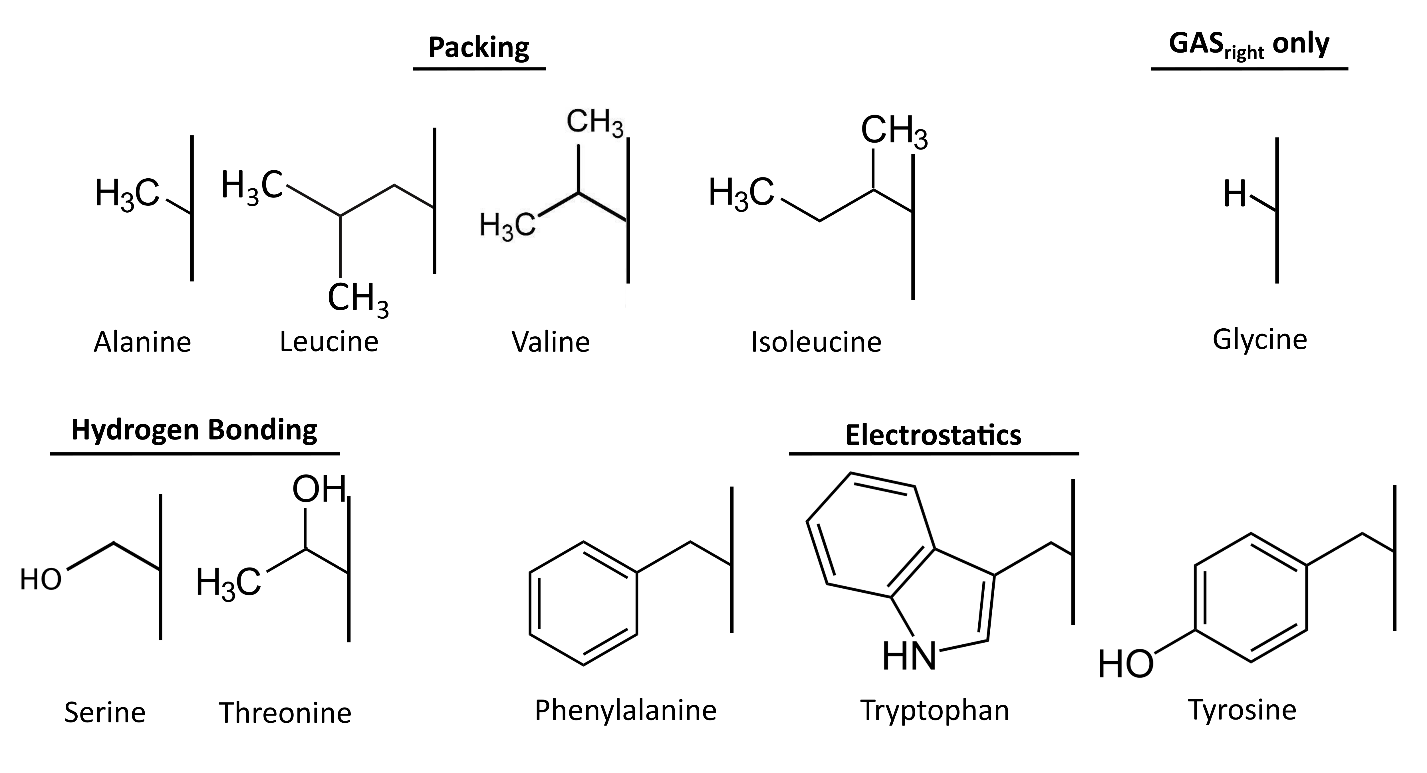
In our design procedure, we included AAs that had the potential to hydrogen bond due to how frequently they are found in membrane proteins (S3.4). Our design energies predicted low levels of hydrogen bonding, suggesting that these AAs would not result in hydrogen bonding impacting association. However, because our energetics show little correlation with experimental dimerization propensity in the Left and Right design regions, it is difficult to confidently state that our sequences associate solely by van der Waals packing without the influence of other forces like hydrogen bonding.

**A graph of different colored bars

Description automatically generated with medium confidence**To ascertain if our proteins associate solely by packing, I conducted an experiment where I mutated our designed proteins to remove the potential for hydrogen bonding. To identify proteins with the potential for hydrogen bonding, I wrote a Python script that searches through my protein structures and identifies any oxygen atoms within 3Å, a generous threshold for potential hydrogen bond formation. I identified 17 proteins that associated from mildly weak to strong dimers (>40% GpA) with the potential for at least 1 hydrogen bond. I mutated all hydrogen bonding AAs in these sequences to hydrophobic AAs with similar steric bulk: Thr->Val, Tyr->Phe, and Ser->Ala. I ordered the original design sequences, their respective clash mutants, and the hydrophobic mutants as gblocks from Twist Bioscience and successfully cloned 13/17 proteins into the TOXGREEN plasmid for experiments.

**Figure 3.13 TOXGREEN on hydrogen bonding AAs.** TOXGREEN fluorescence of 6 biological replicates for each design (WT), clash (C), and hydrophobic (HP) mutations.

8/13 designs were found to properly associate according to our designed interface, where either the clash (C) or hydrophobic (HP) mutations resulted in a noticeable decrease in association when compared to the WT (Figure 3.13). 4/8 of the hydrophobic mutations displayed similar or increased association as compared to WT. Although we are unable to predict association using our energy terms, this result indicates that we have successfully designed proteins that associate without hydrogen bonding. This data suggests that although a weak force, van der Waals packing drives the association of a variety of our designed membrane proteins.

4.3 Studying the impact of sidechain packing with other forces

**Figure 4.2 Chemical Structures of Design AAs.** Design AAs separated by structures and potential energetic contributions to MP folding.

To investigate the impact of sidechain packing on MP folding and association, I designed thousands of proteins using only a subset of AAs (Figure 4.2). However, this subset of AAs includes two AAs (Ser and Thr) with that can form hydrogen bonds (Russ & Engelman, 2000) and three AAs (Trp, Tyr, and Phe) that can facilitate electrostatic π-π stacking interactions (Johnson et al., 2007). Our current dataset has the potential to facilitate these interactions outside of solely sidechain packing, however, our energetics suggest that they associate primarily through packing. Mutational testing on our sequences suggests that hydrogen bonding is unlikely to play a role in all our sequences (section 4.2), and visual inspection of structures does not suggest that electrostatics interactions are involved in association.

We included these AAs because of their relative abundance in MP sequences (S3.4). Ser and Thr are typically found to hydrogen bond with the backbone carbonyl oxygens in monomeric helices (Gray & Matthews, 1984), so we expected the addition of these AAs not to have a large impact on folding through additional hydrogen bonding. Larger AAs (Trp, Phe, and Tyr) have an extensive van der Waals radius due to their size and steric bulk, therefore we wanted to include them in our experiments. Excluding these hydrogen bonding and aromatic AAs would restrict our design pool to four AAs (Ala, Leu, Val, and Ile). Because we decided to use a mutagenesis strategy implementing Ala and Ile, our designs would be restricted with the number of potential designable sequences. Additionally, sequences designed with a small pool of AAs would not be representative of MP-like sequences. However, studying the impacts of forces alongside packing could be addressed using our current methods.

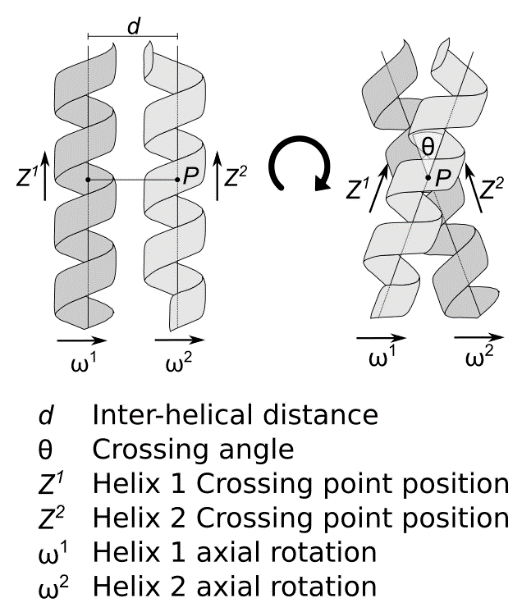
To study the impact of sidechain packing alongside other forces, we can rerun our design algorithm and change how we pick sequences for experiments. I decided on sequences by the calculated van der Waals energy, but a follow up study could instead choose sequences with a considerable amount of hydrogen bonding. We can then see if an increase in the amount of predicted hydrogen bonding and number of hydrogen bonds results in higher dimerization, like GASright. Another option is to include electrostatics in our energy calculation (Patel et al., 2004; Zhu et al., 2012). Including this term may result in designing structures amenable to forming electrostatic interactions. If the energetics are found to match dimerization propensity for structures with more hydrogen bonding or electrostatics, it would suggest that these other forces play a more prominent role in helix-helix association than packing. This would support the data found in my initial study suggesting that packing is a weak driving force. By choosing sequences with a range of packing energies for each of these other energetic variables, we may also be able to identify a trend between packing and its impact on association alongside other forces.

While the previous ideas utilize the same AAs as the designs run in my paper, designing sequences with an alternative set of AAs could also be an option. We can continue to include the AAs that can only pack (Ala, Leu, Val, and Ile) alongside other subsets of AAs. Designing sequences with hydrogen bonding (Ser and Thr) or electrostatic (Phe, Tyr, and Trp) AAs would allow us to better isolate sidechain packing with these forces. Alternatively, charged interactions have been found to impact folding in a variety of MP systems (Ulmschneider et al., 2017). Designing using a subset including the two charged AAs (Arg and Lys) to facilitate association would allow us to determine the impact of packing with different numbers of charged AAs. By designing sequences with similar geometries with these subsets of AAs, we can potentially tune the amount of hydrogen bonding, electrostatics, or charged interactions for each sequence. Similar studies have mutated single residues on helices to determine the effect of different AAs (Choma et al., 2000; Zhou et al., 2000; Zhou et al., 2001). If we find that mutations to particular residues increase or decrease association, we can determine how different forces influence association alongside changes in packing.

We can further assess the impact of sidechain packing on association by altering the backbone sequence. We have primarily used a poly-Leucine backbone in our previous research to assess dimerization of the interface. Another approach is to assess the dimerization for sequences with the same interface on poly-Leucine, poly-Alanine, and poly-Valine backbones. Changing Leu->Val results in the loss of a single CH2, and changing Val->Ala results in a loss of 2 CH3, both reducing the size of the sidechain. Each of these AAs undergoes sidechain packing, however, the loss of steric bulk from Leu->Val->Ala suggests that poly-Val and poly-Ala backbones would exhibit less van der Waals packing. By investigating how these small changes impact association of our design interfaces, we can gain insight into how minute changes in packing result in differences in association. Additionally, these experiments may give better insight into studying the van der Waals packing interactions with the membrane environment. This includes a more detailed understanding of the entropic cost of these AAs on their role in MP stability. Overall, these experiments may allow us to correlate the changes in association with change in AA size, which could contribute to tuning our van der Waals energy term to differences in steric bulk. These experiments may give us insight into how to better predict the van der Waals packing between the membrane and the protein backbone.

4.4 Improving the protein design algorithm

In Chapter 3, I detailed how I developed my protein design algorithm. Although minor changes have been made throughout my research, a majority of the program was built in 2021. Additionally, a variety of machine learning algorithms have been developed that predict protein structures to near atomic precision. In particular, AlphaFold2 revolutionized the way that we look at the puzzle of solving protein structures. My data suggests that I have successfully designed sequences that associate along the designed interface. However, our energetic terms are unable to predict the ability for these proteins to associate. Inspired by the recent literature and thinking of ways to continue the research, in this section I detail ways to develop and build off my algorithm for future research.

4.4.1 Heterodimer design

**Figure 4.3 Heterodimer Geometry.** Heterodimeric sequences do not need to have a symmetric geometry, where the z-shift and axial rotations of each helix can be different from its partner.

My research focused on assessing the stability of homodimer proteins, partially due to their simplicity to design. When designing homodimer sequences, we take advantage of symmetry: Helices are made up of the same sequence and each helix is computationally placed at the same value for each geometric term. However, important biological interactions such as regulating gene expression are carried about by heterodimeric receptor tyrosine kinases (Del Piccolo et al., 2017). I am currently working with another graduate student in the lab to develop our protein design algorithm for heterodimer sequences. Heterodimer design adds multiple variables for design, namely each helix composed of a different sequence and can be placed at non-symmetric geometries. The two most important geometric terms for heterodimer design are the axial rotation and the z-shift. Each of these terms helps to define where the crossing point of the interface is found, with respect to the individual helix (Figure 4.3). This exponentially widens the geometric space for heterodimer association: Instead of a 1-to-1 ratio for each axial rotation versus z-shift, all rotations on one helix must be assessed against all rotations on the other helix, and the A screenshot of a graph

Description automatically generatedsame must be done for z-shifts.

**Figure 4.3 Data separated by groups of %GpA. A)** Data from Anderson et al. 2017, showing that CATM predicts sequences with a higher dimerization propensity to have distinct geometries and energies. **B)** Data from CATM runs on my GASright designs. Similar results are found, with CATM predicting a better energy score, van der Waals, and hydrogen bonding on average for more stable designs, as well as a narrower crossing angle and interhelical distance.

As an initial approach we aim to design heterodimeric GASright sequences. Although heterodimers are not necessarily symmetric like homodimers, it is possible for heterodimers to have symmetric geometries. Therefore, attempting to design heterodimer sequences from known homodimer geometries is a reasonable initial approach. In a previous study in our lab, we found that distinct geometries in GASright result in different levels of association according to our CATM prediction algorithm (Anderson et al., 2017; Mueller et al., 2014). I ran CATM on my designed GASright sequences and created a table of data similar to as found in Anderson et al. 2017 (Figure 4.3). As demonstrated previously, CATM predicts the association GASright sequences and shows that sequences with different levels of association (S4.1) have a geometric dependency. The interhelical distance and crossing angles narrow as dimerization propensity increases. There does not seem to be a large dependency on axial rotation and z-shift, but these values give us A diagram of a dna structure

Description automatically generatedstarting points that can be applied in heterodimer design.

**Figure 4.4 Heterodimer design strategy.** To simplify heterodimer design, we can take known non-homodimerizing sequences (templates) from previous research and design a sequence to associate with it (design).

To simplify heterodimer design, we can design sequences against a single template sequence (Figure 4.4). I expand on how I would implement this into my design algorithm in Supp. 4.6.1. From data in our previous work, we have characterized many sequences that do not homodimerize. These non-homodimerizing sequences are ideal templates to use for heterodimer design. We can use each of these sequences as templates for design, aiming to find sequences that associate as heterodimers. After designing sequences against these templates, we need to ensure that the designed helices do not homodimerize. We can determine this by predicting their homodimerization in CATM (Anderson et al., 2017; Mueller et al., 2014). Any sequences found to associate with a stable energy in CATM can be A diagram of a diagram of a diagram

Description automatically generatedremoved from our pool of sequences that we plan for experiments.

**Figure 4.5 Heterodimer experimental strategy.** By expressing two plasmids that code for different antibiotic resistance (AMP and KAN) and each expressing a different TM sequence (Template and Design), we can investigate the dimerization propensity of heterodimers.

After designing sequences to associate to a template non-homodimerizing sequence, either TOXGREEN or sort-seq can be used to evaluate the dimerization propensity. Other students in my lab are developing a two-plasmid TOXGREEN system, where one plasmid codes for the template sequence and the other for the design. We can clone the template sequence into a bacterial strain with resistance to an antibiotic, then clone a library of our designed sequences into the bacterial strain that expresses the template sequence with resistance to an additional antibiotic (Figure 4.5). Because we are designing GASright sequences which show better correlation to our energetics, we expect that exploring a range of energies of our designed heterodimers will yield a range of dimerization.

One limitation of using homodimer geometries for heterodimer design is the potential lack of sequence diversity at the interface. The symmetrical nature of these backbones may often result in similar (G/A/S)xxx(G/A/S) at the interface. To account for this, we can also study the effect of making point mutations along the design sequence. By mutating interfacial positions to another AA often found in GASright, we can compare dimerization propensity based on small changes in packing (G->A/S) or addition of interhelical hydrogen bonding (A/S->G). If our energetics determine that the main differences between two sequences with one AA difference is mostly due to a loss of van der Waals packing, the data would suggest that packing is a stronger driving force when applied alongside interhelical hydrogen bonding. The energetic data from previous research suggests that this is a possibility (Anderson et al., 2017; Díaz Vázquez et al., 2023), where van der Waals packing contributes to stability more than hydrogen bonding.

To further expand on geometries for heterodimer design, we can explore the stability of helices at various combinations of axial rotations and z-shifts. For homodimers, I assessed the energetics of poly-Leucine sequences with Ala or Gly at all interfacial positions for randomized symmetric axial rotations and z-shifts (Section 3.3.3). We can use this method to identify asymmetric axial rotations and z-shifts. Rather than brute forcing and assessing energetics for x2 axial rotations times y2 z-shifts, we can explore favorable parameters from previously studied homodimeric GASright proteins. Combining the GASright designs in my study with other research in the lab, we now have a database of hundreds of GASright homodimers. By combinatorial testing common axial rotations and z-shifts (Figure 4.3), we may be able to identify regions that are energetically favorable for heterodimer design.

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Description automatically generated with medium confidence4.4.2 Turning sequence entropy into a pairwise term

**Figure 4.6 Amino acid frequency from TMs extracted from OPM.** A)Each AA was counted as found in TMs identified from OPM and divided by the total count of all AAs to determine the frequency. Separated by year that data was extracted. B) Frequency of the pairs found in at least 10 designed sequences compared with the largest differences between designs and all TMs. Most pairs are found more often in TMs (more negative). Pairs found to be all regions marked in red, and pairs found in Left and Right marked in blue.

To design sequences similar to natural MP proteins, we created a SEQUENCE\_ENTROPY term detailed in section 3.3.4. Briefly, this term uses the natural distribution of AAs in MPs to design a sequence similar to MPs. It is currently implemented as a similarity score, with higher values being determined as more similar. We expected this term to help normalize experimental expression and insertion, however, we may not be maintaining packing interactions found in natural MP structures. Previous research has shown that protein activity and folding are affected by small changes in AA sequence (**cites**). To further investigate the impact of packing on these changes, we can calculate how individual AAs might affect those around them.

I recalculated the composition of AAs for all non-redundant TMHs in OPM as of May 14, 2024. The composition is quite similar to when determined in 2021 (Figure 4.6A). I then chose to analyze the pairs of AAs found within the interface of my successful designs (as defined by SASA) and those within the TMHs (Figure 4.6B). Although we were able to successfully design interfacial sequences that reflect the frequency of AAs in TMs (S3.1), these are pairs of AAs that are most different from the frequency in all TMHs. However, this comparison is between all AAs within TMH sequences and not the interfaces of TMs. It may be more informative to first identify interfaces between TMs from solved structures and use this AA frequency for future designs. Additionally, beginning to identify relationships between AA frequency by position (AA3 = alanine, alanine separated by 3 bases) could also benefit designing sequences that maintain atomic interactions found within natural MPs.

4.4.3 Machine learning ideas

One final approach to improving the design procedure is to better optimize our energetic algorithm using machine learning. Another student in the lab is using machine learning to optimize the weights for CATM, aiming to gain a better understanding of which forces contribute more to stability. By applying similar regression training to fit each of our energy terms ("A Review on Linear Regression Comprehensive in Machine Learning," 2020), we may be able to identify the reason why our energetics do not correlate well outside of GASright. This will provide us with the ability to better predict the energetics of sequences designed outside of GASright.

Machine learning could also be applied to improving upon design without reliance on energy terms. Alphafold2 uses multiple sequence alignments (MSA) as a foundation for determining structures from a given sequence (Jumper et al., 2021). By applying similar machine learning algorithms using our database of helical TM pairs, we may find that designing sequences with machine learning is more accurate than how we currently design. Instead of energetics, sequences will be designed based on the similarities between sequence and geometry information from our database. Additionally, most of the sequences in the database of TM pairs are heterodimers. By implementing a machine learning strategy, designing heterodimers may become more tangible without having to rely on searching the immense heterodimer geometric space. For example, we can take many of our TM helical pairs and replace non-interfacial residues with leucine to mimic our poly-Leu based designs. Building an MSA-like database from these TM pairs and applying similar machine learning methods to predict distances between pairs of AAs as AlphaFold2, we may be able to design novel heterodimer sequences. These heterodimers would also not be limited to GASright, expanding the scope of the previous heterodimer design ideas.

4.5 Detecting protein concentration in high-throughput

One of the weaknesses of our TOXGREEN assay is the inability to accurately assess the expression levels of each our proteins in the membrane. Currently, much of the research holds the assumption that our proteins express at the same level. We designed experiments to control for this variable previously by studying the dimerization of interfaces on poly-Leu backbones and in my study by maintaining the sequence composition as found in natural MP sequences. Subsets of sequences are then extracted and analyzed for their ability to express using western blots. Although we found that sequences designed in each region had similar expression, the Right-handed designs displayed noticeably less expression than both Left and GASright (S2.1). Additionally, the western blot only assesses total protein concentration, not considering just the proteins that are inserted into the membrane. Therefore, it is possible that our Right-handed designs express and insert the same in the membrane as other designed sequences. However, we currently do not have the tools to assess this. To improve our accuracy in assessing MP association, developing a way to assess the concentration of our proteins in high throughput would be beneficial, allowing us to normalize the fluorescence yield of each sequence to the protein concentration.

One way to measure the expression of our MPs is to use fluorescence. By labeling our proteins with fluorescent proteins that are viable in bacteria, we can measure the amount of expressed protein through fluorescence. Recently, fluorescent proteins have been used to track movements of membrane proteins within bacterial cells (Lyu et al., 2022; Navarro et al., 2022). Since we measure the fluorescence of expressed superfolder GFP (sfGFP), a molecule expressing a different wavelength of light such as TagRFP-t (tRFP) (Yang et al., 2021) can be expressed in our TOXGREEN cell line fused to MBP on our proteins. We can detect the amount of tRFP signal alongside our sfGFP signal on a FACs. However, the total fluorescence output from tRFP would relay the same information as western blots. Instead of just reading the tRFP information for each counted cell, we can set the FACs to identify an acceptable window of fluorescence; Cells that express beyond the upper and lower bounds of tRFP fluorescence would not be counted. Some sequences may inherently express more than others, so we can run multiple sorting runs on populations with different bounds of tRFP. This would allow us to measure the dimerization propensity of proteins with similar levels of MP expression. Finally, we could normalize by the amount of TagRFP-t and assess dimerization propensity against expression.

4.6 Supplementary Details

Below, I detail the changes I would make to the design code in order of priority (**maybe make this a figure; could also directly reference pieces of my code?**):

1. Separate helices: one as template and the other as designed
   * Add an additional option for the template helix so that an input sequence can be given on command line or in the configuration file
   * Set both helices to the input sequence, then only add AAs for design to the design helix
   * Adjust the (add function here) to only change the AA on the design helix
   * Check that the BASELINE\_MONOMER still works for heterodimers (if not, adjust it)
2. **Backbone optimization changes** 
   * **Look into the code**

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