Introduction to biomolecular visualization: When do we call a polypeptide a protein?[*]

Biochemistry textbooks use the term *protein* in the first chapters as if we all know exactly what that means. For example, Chapter 1 of Moran, et al.[1] states that "a functional protein can be a single polypeptide or it can consist of several distinct polypeptide chains that are tightly bound to form a more complex structure." Going further into this definition, a *polypeptide* is a sequence of *amino acids* joined by *peptide bonds*.

Going further into this definition, a polypeptide is a sequence of amino acids joined by peptide bonds. The International Union of Pure and Applied Chemistry (IUPAC), the arbiter of terminology in chemistry, define a polypeptide as a "peptide containing 10 or more amino acid residues." They then define proteins as "naturally occurring and synthetic polypeptides having molecular weights greater than about 10000 Da (the limit is not precise)." Because the average molecular weight of an amino acid is about 110 Da, the IUPAC definition implies that the smallest proteins consist of ≈100 amino acids. Others have set boundaries at 50 or 20-30 amino acids. And definitions based on counting amino acids don't account for the word "functional" in the textbook definition. So what characteristics should a polypeptide have to be considered a protein?

In 2008, Honda, et al.[2] published a claim that a synthetic polypeptide with 10 amino acids should be considered a protein. We'll use that paper and its supporting information as a starting point for our own consideration of whether a particular polypeptide can be considered a protein. Here are our specific goals.

1. Review the experimental and computational evidence that Honda, et al. present for their conclusion.

2. Reproduce the structural analysis of the 10 amino acid protein using the molecular visualization software VMD.

3. Explore the RCSB Protein Data Bank to find other small polypeptides that can be studied using the same kind of analysis.

4. Choose two peptide toxins and develop evidence that supports whether those toxins should be considered as proteins.

Pre-experiment work

These questions and guidelines should help shape what you include in your lab notebook for your pre-lab work. In addition, be sure to address any other items in the general statement about pre-lab guidelines for any biochemistry experiment.

1. Read what the first chapter or two of your biochemistry textbook says about proteins. Summarize what the textbook implies are the key characteristics of proteins.

2. Read, in the following order, the Introduction, Discussion, Figures 1 and 2, and Results (skipping the section "Structural Dynamics of CLN025") of Honda, et al. Summarize the authors' work being sure to address the following questions.

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a. What are the sequences of the original polypeptide chignolin and the CLN025 peptide that this paper focuses on?

b. What *experiments* do the authors use to make a case that CLN025 has protein characteristics and chignolin does not? What are the results of those experiments?

c. What *structural analysis* do the authors use to support the case that CLN025 has protein characteristics? (Do not include any of the structural dynamics work.)

d. Comment on the how well the author's data and results support their conclusions.

3. Use the RCSB Protein Data Bank (RCSB PDB) at http://www.rcsb.org and search for CLN025. Find the entry that corresponds to the crystal structure submitted to the Protein Data Bank by the authors. Use the information in that entry to obtain the following information.

a. The PDB ID of the CLN025 crystal structure.

b. The resolution of the crystal structure (with correct units).

c. The classification of this macromolecule. Include a definition of the term used.

d. The amino acid sequence, verifying that it is the same as in the Honda paper.

e. Any secondary structure present.

4. Find all the PDB IDs for CLN025 and chignolin entries. Note whether each corresponds to a crystal structure or NMR structure.

a. *Read Section 4.2 of Principles of Biochemistry and summarize the similarities and differences between the information we obtain from crystal structures and NMR structures.*

Visualization and analysis

We first work on reproducing the CLN025 structural analysis of Honda, et al. We will use the molecular visualization software VMD,[3] available at http://www.ks.uiuc.edu/Research/vmd. Any current version of VMD should have all the key components for this experiment. You should download and install VMD now if you're using your own computer.

The protocols in this section are based on Hsin, et al., "Using VMD – An Introductory Tutorial."[4] You may wish to refer to that article for additional details and additional protocols for other kinds of biomolecular analysis that you can perform with VMD.

Your research team should divide up into pairs for this work on the protocols. Each pair should complete the same protocols on two different computers, including both sets of results in the team notebook. The final report will require the team to choose which set(s) they wish to include. Each team member must become familiar (in the hands-on sense) with the tools and protocols.

Loading, displaying, and manipulating a molecule

In order to display a molecule, VMD (and any visualization program) needs at least three sets of information:

- The identity of each atom in the molecule.

- The coordinates in Cartesian space (*x*, *y*, *z*) of each atom.

- The other atoms each atom is bonded to.

For files from the Protein Data Bank, this information is stored in a standard format known PDB. Many other formats exist, but you won't have to worry about those for this experiment.

We will therefore load this information into VMD and then let it do its magic in order to display the structure.

Protocol 1: Loading and initial display

A. Start a VMD session.[*] Two windows will appear. The **VMD Main** window has many of the commands that we will us in menus and will display the list of all molecules that we have loaded. The **OpenGL Display** window (which we'll call the Display window) is where all the graphics appear.

B. In the Main window, choose File→New Molecule…. The **Molecule File Browser** window will appear. You will choose one of the following two ways for

    a. Version 1.94a of VMD can load files from the RCSB Protein Data Bank directly by using the PDB ID. For the Filename, enter the PDB ID for the BSA crystal structure (5AWL) that you found in the pre-experiment activities, then click Load.

    b. If you're using Version 1.93 of VMD, do the following more complicated set of steps.

        i. Click on the Download Files button on the PDB page for 5AWL and select PDB format. That will create a file 5AWL.pdb on your computer.

        ii. In the Molecule File Browser in VMD, click the Browse button and open the 5AWL.pdb file. Then click on Load.

C. You should now see a line structure of CLN025 in the Display window. Close the Molecule File Browser window.

Protocol 2: Manipulating the display

A. Click (on Windows, that's click with the left mouse button) and drag the line structure to see that you can easily rotate the molecule in the window.

B. Now click and drag with the right mouse button (on the Mac, hold down the Command key and click) to see that you can perform a different kind of molecule rotation.

C. Click on the Mouse menu in the VMD Main window. Note that the mouse is currently in Rotate mode. Change the mode to Translate and click and drag using both the left and right mouse buttons to see how you can move the molecule in the window.

D. Go back to the Mouse menu and choose the Scale mode. Click and drag with the left mouse button to see how you can change the size of the molecule.

    a. You can use keyboard shortcuts—*r, t, s*—to switch between these modes without going to the Mouse menu.

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[*] If you're running VMD for the first time on a Mac, hold down the control key and click on the VMD icon, then choose Open. In the dialog box that appears, choose Open again. You'll only have to do this step this one time.

E. When a molecule is first loaded, VMD will rotate it about a point in the center of the molecule. From the Mouse menu, choose Center, or press *c*, and then click on an atom in the molecule. Return to Rotate mode, and you should now see that you are rotating the molecule about the atom you clicked on.

F. If you ever want to return to a default view, press = on the keyboard (or select Display→Reset View in the VMD Main window).

G. Let's do two other quick steps to make the display look a little better. First, choose Display→Orthographic in the VMD Main window. When you're first looking at structures, this viewing choice is a little easier to interpret. Second, we don't care about the axes being displayed, so turn those off by choosing Display→Axes→Off.

H. For the final task in this section, choose Mouse→Query in the VMD Main window (or press 0). Then click on any atom. It may not seem like anything is happening. But go look for the third window that VMD opened. It will contain all kinds of text information about what VMD is doing. When you click on an atom while in Query mode, you get lots of information about that atom. Find examples of each of the following kinds of atoms in this structure and for each, give the following information that VMD reports: the name of the atom, the index (how VMD numbers the atom in the molecule), the residue name the values of residue and resid[*].

    a. An α-carbon (the name will be CA)

    b. An oxygen on a tyrosine

    c. A sidechain nitrogen on a tryptophan

    d. An oxygen in a water molecule (look carefully for these; if you have trouble finding these, return to this step after you do Protocol 3)

Protocol 3: Changing the molecular representation

When you first load a molecule, you will see the line structure. VMD allows you to modify the display of those lines as well as choose many other ways to display the molecule, each of which is called a representation. Those methods are not just to make the molecule look good; they are helpful in our analysis. We'll explore several key methods in this section.

A. Choose Graphics→Representation from the VMD Main window. You'll get a new window with the title **Graphical Representations**. The pop-up menu will contain the name of your molecule. You only have one representation right now; when you have more, the one you're editing is highlighted.

B. In the current line representation, you can change one parameter, the thickness of the lines. Play with that value until you find a thickness you like.

    a. *Optional* From the Drawing Method window in the Graphical Representations window, select Licorice. This representation is a little nicer, but it can also be a little slower to manipulate. Any place where the Lines representation is mentioned, you

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[*] "residue" is the number that VMD assigns to the amino acid, solvent molecule, or ligand, starting with 0 for the first residue in the structure. "resid" is the number assigned to the same species in the original PDB file. Those numbers are typically not identical.

can use the Licorice representation instead. (And the water molecules are a little easier to see in this representation.)

C. Let's add a second representation. First, click on Create Rep in this window. You will see that a second, identical representation appears. In the Drawing Method pop-up menu, choose VDW. What do you now see? Choose a Sphere Resolution you like (but don't go above 30).

D. Demonstrate that you can turn the VDW representation off and on by double-clicking it in the Graphical Representation window. Being able to turn representations off and on is a very valuable analysis tool.

E. Turn the VDW representation off, create another representation, and choose NewCartoon for the drawing method. Then, in the Coloring Method pop-up menu, choose Secondary Structure. You may also want to turn off the Lines representation.

   a. To determine the kinds of secondary structure present in CLN025, we need to interpret the colors. In the VMD Main window, choose Graphics→Colors. In the **Color Controls** window that appears, select Structure from the Categories list. In the Names list, you'll see each kind of structure that VMD knows about and its corresponding color.

   b. List the different kinds of secondary structure present in CLN025. Is the secondary structure information VMD displays consistent with what you found in the pre-experiment questions?

F. Now just display the Lines (or Licorice, if you chose to do that) representation. When a protein crystallizes, it often incorporates solvent molecules and other solutes into its structure. To see that here, select that representation, replace the word *all* in the Selected Atoms field with *not protein* and press enter. You should now see a bunch of red dots. (And you may need to turn off all the other representations or use the Licorice representation to see those.)

   a. The red dots are the oxygen atoms of all the water molecules in the structure. So it's now a good time to research the answer to this question: Why aren't hydrogen atoms typically present in protein structures determined by x-ray crystallography?

G. We won't be interested in anything that's not the protein for the moment, so let's turn off all the other molecules. Do the following for each representation. Select the representation and change the Selected Atoms field to "not water". Then press Enter. Be sure to turn each representation on as you make the change so that you can see the effect. For the Lines and VDW representations, it should be clear that the water and small molecules are no longer displayed. We'll work more with atom selections later.

H. One other value representation shows the volume the molecule occupies in a way that is more accurate than the VDW space-filling mode. Leave only the Lines (or Licorice) representation on. Then, create a new representation and choose Surf as the Drawing Method and ResType as the Coloring Method. Verify that "not water" is still the selection. As you did with the secondary structure coloring method, determine which

residue type each color corresponds to. Based on your knowledge of amino acid types, verify that each amino acid in CLN025 has been assigned the correct type by VMD.[*]

I.   The last task of this section is to use the tools introduced so far to create an image of CLN025 that your team likes. That image should use more than one representation, but otherwise you have your choice of drawing methods, coloring methods, and materials. To save the image in the Display window, select File→Render in the VMD Main window. You can just click on Start Rendering to get a snapshot of the Display window, but you might also try selecting "Tachyon (internal, in-memory rendering)" in the first pop-up menu and see what you get.

You should save an image of any significant visualization you've done. For example, you will be asked later to highlight key sets of residues in the BSA structure. Each time you do that, you should save an image and put it in your lab notebook.

J.   Before you go on, turn off all the representations except the Lines representation.

Interlude: Information available in the PDB

So far, we've only looked at the Structure Summary tab on the 4F5S PDB page. Several other tabs on that page will be useful to us as well. Return to that page and complete the following steps.

A.   Review the following elements from the Structure Summary tab that you have seen previously in this activity.

   a.   Experimental data snapshot: Method and resolution

   b.   Literature reference

B.   From the Macromolecule Content and Macromolecules section in the Structure Summary tab, record the following information.[†]

   a.   Total structure weight

   b.   Number of chains and the letters corresponding to those chains

   c.   Sequence length (and define this quantity)

Protocol 4: SequenceViewer and selecting residues

VMD provides a very nice tool called SequenceViewer that allows us to highlight specific residues.

A.   Open SequenceViewer by choosing Extensions→Analysis→SequenceViewer from the VMD Main window. The **VMD Sequence** window will appear.

B.   This window contains a list of amino acids in the molecule and a color-coded structure column.[‡] Click on any amino acid to see it highlighted in the VMD Display window. The Graphical Representations window now contains a new representation. The atom

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[*] You may find it useful to change the Material of the surface representation to Transparent and turn on the Lines representation.

[†] There is also a "Protein Feature View" subsection, which is way cool, but beyond the scope of what we're doing in this experiment.

[‡] We're not going to use the B value column in this experiment.

selection for that representation is controlled by what you select in the VMD Sequence window, but you can change its Drawing Method and Coloring Method. Do so to get something that you like.

C. You can select multiple residues by holding down the shift key while clicking. Do that in order to highlight the turn structure in the Display window.

Protocol 5: Saving your work

A. You can save a file of commands that VMD will use to reproduce your visualization as precisely as it can—loading molecules, choosing representations, and setting the parameters for those representations. Start by selecting File→Save Visualization State… from the VMD Main window. Enter a filename that ends in the extension .vmd and does not contain any spaces, like "cln025first.vmd", and save that file in a place you can find it.

B. Quit and restart VMD. Choose File→Load Visualization State…, navigate to the file you saved in the previous step and click Open. You should see something very close to what you had when you saved your work.

Throughout this experiment, you should save images and visualization states whenever you have done something that has any possibility of ending up in your final report.

Using VMD to analyze molecular structure

Almost none of what we've done so far helps us reproduce the work of Honda, et al. That's about to change. VMD (and any similar program) has powerful measurement and analysis tools that provide a great deal of structural insight. That makes it best to restart VMD so that we have a blank slate.

Protocol 6: Distances, bond angles and dihedral angles

A. Start with the CLN025 structure with only the Lines representation turned on. We'll start with the default Lines representation. And also be sure to display only the protein atoms.

B. Choose Mouse→Label→Atoms in the VMD Main window. Click on any atom and a label with the residue name, number, and atom name will appear (although not always be easy to see). Clicking on the same atom turns the label off. Label three atoms in this way.

C. Choose Graphics→Labels in the VMD Main window to bring up the **Labels** window. Select Atoms in the pop-up menu if it's not already selected, and you'll see the list of atoms you've labelled. Black means a label is on; red means it's off. If you click on any label, you'll find all the information about the atom that we saw earlier in this experiment. (You can also show, hide, and delete labels from this window.)

D. Let's go back to the VMD Main menu and choose Mouse→Label→Bonds. Now click successively on two non-bonded atoms that are near each other. You'll see the atoms connected by a dashed line that is labelled by the distance in Å. Sometimes that's hard to read, but no worries! Just go to the Labels window and choose Bonds from the pop-up menus to see all the distance labels you've made. Each label has the distance stored in the Value field.

E. We'll also be analyzing dihedral angles. Choose Mouse→Label→Dihedrals in the VMD Main window. Now click on four bonded atoms in succession—the first atom should be

bonded to the second atom, the second atom should be bonded to the third atom, and so on. The value of the dihedral angle will now be displayed.

F. Now (and finally!) we can examine some of the results from Honda, et al. Let's start with Table S3 in the Supporting Information for the paper. This table lists several dominant interactions in the CN025 structure. Measure the distance between each listed pair of atoms. Divide this work up among the two pairs in your team. You may also wish to display a transparent VDW representation to help you see how these atoms interact.

G. Figure S3 in the supporting information gives values for the two backbone dihedral angles and two sidechain dihedral angles for each residue in the CLN025 crystal structure. Each pair in the team should choose one residue (but not Gly 7 because it has no sidechains) and verify the values in Figure S3.

H. Reproduce image A in Figure S.5 as best you can. There will be some differences since Figure S.5 is based on the NMR solution structure, not the crystal structure. A hydrogen bond is of the form A…H-B where A and B are highly electronegative atoms, typically N or O in biomolecules. The A-B distance in a hydrogen bond is usually less than 3.5Å although some weak hydrogen bonds may have an A-B distance up to 4Å. Given this criterion, does the crystal structure have the same hydrogen bonds in this region as the NMR solution structure? (And if you really want to test your VMD skills, try reproducing S.5C.)

Comparing molecules

All major molecular visualization programs provide many tools to compare molecules. We'll use some of those tools to reproduce Figure S4 in the supplementary information of Honda, et al. We will learn how to do three tasks: Loading multiple molecules, selecting a subset of atoms or residues in those molecules, and aligning the molecules so that we can see similar structures.

Protocol 7: Loading multiple molecules

Figure S4 compares both the crystal and NMR solution structures of CLN025. As noted in Section 4.2 of *Principles of Biochemistry*, NMR does not give us a single structure. We instead get an ensemble of structures that are consistent with the observed data and therefore represent likely structures present in solution.

So we'll want to load both structures into VMD. When we do that, each structure becomes its own molecule with its own set of representations. Let's see how that works.

A. Launch (or re-launch) VMD so that you have a clean workspace to start. Load the crystal structure of CLN025 like you did in the first part of this experiment.

B. With the Molecule File Browser window open, select "New Molecule" in the "Load Files for" pop-up menu. Then, load the PDB ID that you identified in the pre-experiment work as that for the NMR solution structure of CLN025 (2RVD). A second molecule appears in the VMD Main window, but it now has 20 frames. Use the player slider at the bottom of the window to scan through those frames and get a sense of what is the same and different about the 20 structures.

C. When loading multiple molecules, they sometimes are not in the same region of space. Use the Scale mode to zoom out so that you can see both the solution structure and the

crystal structure. We'll get them into the same place in a little while. But for now, hit the = key so that we're focusing on the solution structure.

Protocol 8: Selecting a subset of atoms

A. In looking at Figure S4, we see information about how the authors aligned the different structures to produce the figure. We'll practice doing that using the Graphical Representations window so that we know we're selecting the right atoms. Choose Graphics→Representations from the VMD Main window to open that window and use the pop-up menu to select the solution structure if it's not already selected.

B. Quite often, we are just interested in the backbone of the polypeptide. If we just want to view those atoms, we can change the Selected Atoms from "all" to "backbone" and press enter. Our representation changes to show only the backbone atoms.

C. Suppose we only want to look at the tyrosine amino acids. Enter "resname TYR" into the Selected Atoms field and press Enter. You'll see only the three TYR residues.

D. We can also examine a single residue by using its resid value. Enter "resid 1" in the Selected Atoms field to see just the first tyrosine. If we want to look at a few residues, we can enter each number like "resid 3 5" or a range like "resid 3 to 7". Try those two selections to see what happens.

E. We can also combine these selections. If we want the backbone atoms of the turn in this polypeptide, we would enter "backbone and resid 3 to 7" in the Selected Atoms field.

F. Many, many more selections are possible. Click on the Selections tab in the Graphical Representations window and you'll see all the keywords that VMD understands.

G. Change the representations so that the crystal structure has "not water" as its selected atoms and the solution structure has "noh" (don't show the hydrogen atoms).

Protocol 9: Aligning different molecules

When we ask VMD to align molecules, the program finds the best possible orientation of the structures that minimizes the distance between equivalent atoms in each structure. We therefore have to have a 1:1 correspondence between atoms in each structure. For example, we could align a structure based on two tyrosine residues in different molecules, but we can't align a tyrosine and an alanine. We'll therefore tell VMD a selection of atoms that we want to use for the alignment that we know represents the same kind and number of atoms in each molecule.

The tool we'll use is the RMSD Trajectory Tool.

A. Launch this tool by choosing Extensions→Analysis→RMSD Trajectory Tool from the VMD Main window.

B. The text box in the upper left contains the atom selection we want to use for the alignment. At the bottom are the molecules we want to align. By default, that selection is "protein". But if we click "ALIGN" (try it!), we get an error message because the solution structure has hydrogen atoms and the crystal structure does not. So let's do a subset that will work.

C. Figure S4A aligns on the residues Asp3-Gly7. So let's try the selection "resid 3 to 7 and noh" to make sure we don't include the hydrogen atoms. You should now see both the

solution and crystal structure with some clear overlapping atoms. To make this more evident, adjust both representations so that you'll be able to differentiate between the solution structures and the crystal structures.

D. Now display only the backbone in each structure to be consistent with the figure.

E. We're just showing one of the possible solution structures, but we can in fact see all of them. Use the solution structure as the Selected Molecule in the Graphical Representations window. Then select the Trajectory tab. At the bottom, there is a Draw Multiple Frames field. Replace "now" with "0:19" (VMD starts counting with 0). You should see all the solution structures in addition to the crystal structure.

F. Reorient your molecule display so that it looks as close to Figure S4A as you can make it. Don't worry if you can't make it look exactly the same. We may not be aligning the molecules in exactly the way they've done.

G. Now let's reproduce Figure S4C. In this case, they have aligned four atoms (N, C, CA, and CB—three backbone atoms and the first sidechain carbon) for Asp3. The selection representing those atoms is "resid 3 and name N C CA CB". Make that selection and use change the representation for each structure to "resid 3". How well does the resulting alignment reproduce Figure S4C?

H. You now have enough information to reproduce Figures S4B and S4D, so do so.

I. What conclusions do the authors draw from this kind of alignment work? Based on your analysis of the alignment, do you agree with their conclusions?

There is so much more we can do with VMD, and the tutorial by Hsin, et al. can help you discover some of the additional tools. But what we've done so far is enough to reproduce the structural analysis of CLN025 by Honda, et al. and give you a resource that we'll apply to biomolecule analysis throughout this course.

So really, when do we call a polypeptide a protein?

The RCSB Protein Data Bank contains structures of all kinds of molecules from small polypeptides to large protein complexes with DNA and RNA thrown in as well. Your team's task will be to apply the ideas of Honda, et al. to other polypeptides that you will be able to find in the Protein Data Bank. We will focus on a subset of animal toxins that are single-chain peptides with 30-70 amino acids. These toxins have generated a great deal of interest, both in finding antitoxins, but also in whether these provide an avenue to therapeutic drugs.

Your team's task is to take two toxins from the lists below, one each from the x-ray structure list and the NMR solution list. Your team will then perform the kinds of analyses done by Honda, et al. and that you've reproduced for CLN025 to develop an argument about whether your team's polypeptide should be considered a protein. (Divide up the work!) As part of this work, be sure to identify all secondary structure and tertiary structure in your toxins. And also be sure to generate and save any images that will help support the case your team makes in the final report.

The lists of peptides include only the PDB ID. You will use that to search the RCSB PDB to learn the details of the toxin. Based on the initial information you find, choose a toxin with an

NMR solution structure and a toxin with an x-ray structure that your team finds interesting. Do not choose the same toxin for either as any other team.

NMR solution structure	X-ray structure
2LR9	4LFS
1ROO	3E8Y
1Q2K	1PEN
2GX1	4TTL
1WT8	
2NAJ	

Explore the PDB data entry for your team's chosen toxins. In particular, record the following information.

- Summarize the biology of the toxin. What species produces it? What does it target in causing its toxic activity? What is the scientific interest in the toxin? (All of this information should be available in the literature reference available in the PDB entry.)

- Record the sequence. Note any secondary structure information identified provided in the PDB entry.

- Load the structure into VMD and describe what you observe. In particular, identify any disulfide bridges present in the system.

Now, you can start performing the Honda, et al. analysis. Because CLN025 does not have a disulfide bridge, you will want to think further about how the presence of those affect whether the toxin is considered to be a protein.

Your team's final report will discuss your work in reproducing the CLN025 analysis and the methods, results, and conclusion for the work on your team's toxins.

References

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