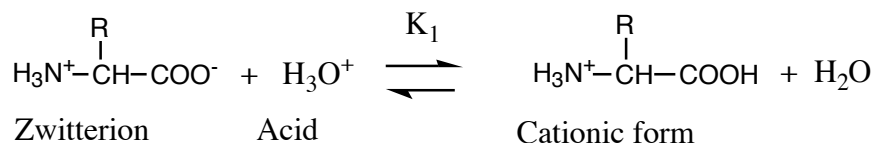


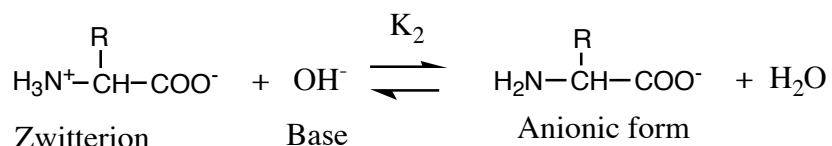
BC367 Experiment 1 Identification of an Unknown Amino Acid

Introduction

As the building blocks of proteins, amino acids play a key cellular role in structure and function. Proteins themselves participate in nearly every physiological event in the cell. In order to understand acid-base properties of proteins and their behavior as polyionic macromolecules, we will begin by investigating the properties of their constituent amino acids. Since all amino acids contain at least one amino and one carboxyl group, they are classified as amphoteric substances (meaning that they can act as either an acid or as a base). Such a molecule reacts with acids as follows:



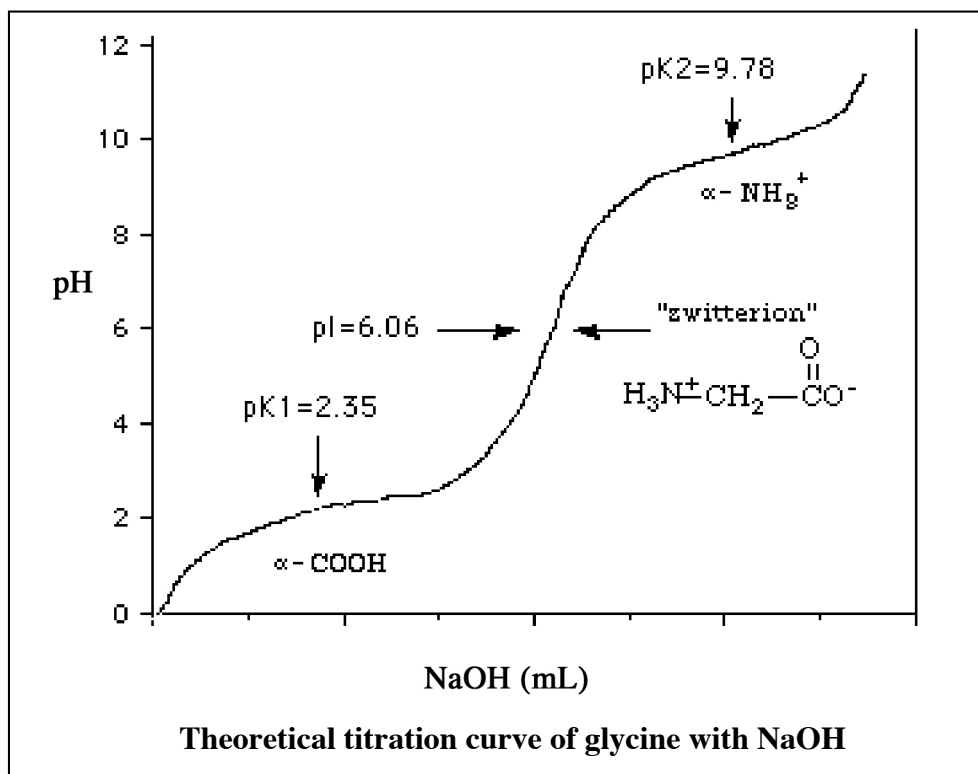
and with bases as follows:



The ionic form of the amino acid present in an aqueous solution depends on the pH of the solution. In this experiment, you will identify an unknown amino acid through acid-base titration. Titration curves of amino acids are very useful for identification. As you can see in the example for glycine shown below, a simple amino acid has two dissociation steps corresponding to loss of H^+ from the acidic carboxyl group at low pH followed by loss of H^+ from the more basic amino group at high pH. The pK_a value for each dissociable group of an amino acid can be determined from such a titration curve by extrapolating the midpoint of each buffering region (the plateau) in the titration curve. The diagram also shows that there is a point in the curve where the amino acid behaves as a "neutral" salt. At this pH, the amino acid is predominantly a zwitterion with a net charge of zero. This point of the titration curve is the "isoelectric point" (pI) and can be approximated as halfway between the two points of strongest buffering capacity (the two pK_a values). The isoelectric point (pI) can be estimated by:

$$\text{pI} = 1/2 (\text{pK}_1 + \text{pK}_2)$$

where K_1 and K_2 are the dissociation constants of the carboxyl and amino groups, respectively. pK_1 of glycine is 2.35; pK_2 is 9.78. Thus, the $\text{pI} = 1/2 (2.35 + 9.78) = 6.06$, meaning that at pH 6.06, glycine has no net charge.



Charged amino acids have acidic or basic side chains (R-groups) giving them more than two dissociable H^+ ions. For example, glutamic acid has a carboxylic acid side chain in addition to its α -carboxyl and α -amino groups. A titration curve for glutamic acid will be somewhat more complex than that for glycine. Three plateau regions and three pK_a values will be observed for glutamic acid: two in the acidic pH region, pK_1 (α -carboxyl group) = 2.2; pK_2 (γ -carboxyl group) = 4.3; and one in the basic pH region, pK_3 (α -amino group) = 9.7. Members of the basic family of amino acids, such as lysine, will also exhibit three pK_a values; however, due to the extra amino group they will have one pK_a in the acidic pH region and two pK_a values in the basic pH region.

The pH at which the net charge of an amino acid is zero is called the isoelectric point, or the pI. To determine this value, average the two pK_a values that flank the neutral species. Thus, the pI for glutamic acid = $1/2 (2.19 + 4.25) = 3.22$. At the pI, the α -carboxyl group is a negatively charged carboxylate ion, the α -amino group is a positively charged ammonium ion, and the γ -carboxyl group is a neutral protonated acid.

Thus, titration curves are helpful in the identification of amino acids as follows:

1. The number of pK_a values differentiates polar and nonpolar amino acids from charged amino acids.
2. The position of the pK_a values for charged amino acids allows one to identify positively charged from negatively charged amino acids.
3. Comparisons between experimental and literature pK_a values can allow the identification of a specific amino acid.

Experimental Procedure

You will perform automated titrations of both a known standard (0.1 M glycine) and an unknown amino acid (also at 0.1 M) with 1.0 M NaOH. You will use your data to determine the pK_a and pI values of each amino acid, thereby allowing you to deduce the identity of your unknown (and those of your classmates). Be sure to note the code number of your unknown IN YOUR NOTEBOOK. A list of possible unknowns will be posted in the lab.

Follow the instructions for the Vernier LabPro device (see the appendix) to perform your titrations. As directed, bring the pH of your amino acid solution to ~ 1.5 before you begin titrating, then titrate with 1.0 M NaOH. (All amino acid solutions have been brought to a pH of ~ 7 for this experiment.)

If you think you have overlapping peaks for your unknown, repeat the titration with 0.1 M titrant. If the area of overlap is at high pH, start with the prepared amino acid solution at pH ~ 7 and titrate up with 0.1 M NaOH. If the area of overlap is at low pH, start with the prepared solution at pH ~ 7 and titrate down with 0.1 M HCl. This may help you to resolve fine detail about the positions of the overlapping peaks. (You can tell for sure whether you have two or three titratable groups after the first titration of your unknown by calculating the approximate number of equivalents needed to reach pK_{a1} and pK_{a2} .)

Analysis

The pK_a values of weak acids can generally be determined from titration curves with good accuracy. An equivalence point is often distinguished by a sharp rise in the slope of the titration curve or a peak in the derivative plot. An equivalence point is preceded by a plateau region where little change in pH is observed with the addition of acid or base. From an inspection of the Henderson-Hasselbalch equation,

$$pH = pK_a + \log [A^-]/[HA]$$

it can be seen that $pH = pK_a$ at the 50% point of a titration because $[\text{conjugate base}]/[\text{acid}] = 1$. The 50% point of titration of a particular species should correspond to the midpoint of the plateau region. An amino acid will have at least two titratable species (the α -carboxyl and α -amino groups) and possibly three (an extra carboxyl or amino group in the side chain). Thus, two or more plateau regions should be observed, the midpoints of which correspond to the pK_a values of the titratable groups.

Note that when titrating a molecule that contains more than one ionizable H^+ , the plateau region for each ionizable H^+ present will be distinct if their pK_a values are more than two pH units apart ($K_1 > 100 K_2$). If the pK_a values differ by less than two pH units, the titration curves will be poorly defined and may overlap to the extent that there is no point of inflection between the two curves; that is, the titration of the second species begins before the titration of the first is complete. Such an overlap of titration curves can usually be detected because of an unreasonably long plateau region that consumes two molar equivalents of titrant. Therefore, you should make sure that you calculate the equivalents of titrant added to help substantiate the identity of your amino acid. You will then have to eyeball the pK_a values of overlapping plateaus.

In addition to the pK_a values, calculate the isoelectric point (pI) of your unknown. Compare the pK_a and pI values of your unknown amino acid to literature values. On your titration plot in

your notebook, label the species found on each region of the curve. Put an annotated copy of your unknown amino acid, with your name and unknown number clearly noted, on the fileserver within 24 hours after the completion of the lab period.

For your write-up, include a graph for glycine and each unknown in your notebook with your assignment of its identity, points of interest labeled on the curve, and the chemical structures of the species found on each region of the curve. In your discussion thoroughly justify the identity of each unknown amino acid on the basis of the titration data. If you had a mixture of unknown amino acids, how might you identify its components?

Appendix: Instructions for the Vernier LabPro

INTRODUCTION:

The Vernier LabPro is a versatile data collection interface that can be used in many different ways in the classroom and in the field. In this experiment, the Vernier LabPro is connected to a computer and used for an automated titration of your amino acids. The titration is monitored via changes in pH using a pH electrode.

INSTRUCTIONS FOR USE:

- A. Make sure that the stir plate and the power supply for the DCU pump unit are plugged in and on. A blinking green light occurs when the interface has power. The Vernier system will complete series of (7) beeps as the system completes a “self check”. If no other beeping sounds are given by the interface, then the system is ready for use. The pump should be connected to the DIG/Sonic1 port and an electrode amplifier with an attached pH electrode should be connected to CH1.
- B. Calibration of the pump (finding the volume of one drop delivered by the pump):
 1. Open the “LoggerPro” program on the dock and then open the “LoggerProExperiments” folder on the desktop. Double click on the icon “**one minute pump cal.cmb1**”. The pump will click and a blinking green light(s) will be visible on the interface when recognized by the software. (The toggle switches on the pump should always be set on the “**computer**” and “**DCU**” settings for the software to communicate to the pump. This should already be taken care of.)
 - NOTE: If the equipment is not set up properly, a “**Sensor Confirmation**” window will be displayed. Be sure the sensors are securely attached to the interface and in the correct ports. If the window is not displayed, continue on.
 - The main calibration window should be activated and the “**Collect**” button found in the top menu bar will be colored green. The table to the left will record collected data in the appropriate columns. The smaller box below the table will count the pulses delivered by the pump. The graph will display a green signal for each pulse.

2. To prime the pump with deionized water, find the inlet tubing (marked as IN on the pump) and submerge the tubing into a clean 100-mL beaker of fresh dH₂O. To prevent air bubbles from entering the line, make sure that the line is secure and that the opening of the tubing is at the bottom of the beaker. Put the outlet tubing into a waste beaker.
3. Click on the green “**Collect**” button. A window will appear that will ask what you would like to do:
 - *erase and continue: will erase the last set of pulses and start at zero
 - *cancel: cancel or exit
 - *append to latest: will add another series of pulses to the last one
 - *store latest run: save data in the table before starting another run

➤ NOTE: **Each run is a series of 30 pulses.**

4. Click on “**erase and continue**”. The pump will run for 30 pulses (refer to the small box under the table). Click on “**Collect**” again and then “**append to latest**” to add another series of 30 pulses. It will take about 90 pulses to prime the pump with water. Check for bubbles in the tubing. If you see bubbles, check your set up and add another set of pulses. If no bubbles are apparent in the lines, then continue.
5. To calibrate the pump (volume in μL delivered by each pulse) find the mass of a clean dry weigh bottle (no top) or small beaker to 0.1 mg. Use a clean, dry Kim Wipe to handle the bottle because fingerprints and dirt will add to the mass and result in calibration error. Set the outlet tubing into the weigh bottle and deliver 60 pulses of deionized water to the bottle. Mass the bottle again. Using the density of water at room temperature (0.9982 g/ml at 20°C), convert the mass of the water delivered to the corresponding volume of water delivered. Calculate the volume of water delivered in a single pulse by the pump (in μL).
6. Remove the inlet tubing from the beaker of water. Empty the line by delivering 30-60 pulses (of air). Place the outlet tubing into a waste beaker and the inlet tubing into a 100 mL beaker of 1.0 M NaOH (or other desired titrant). Prime the pump with NaOH. Be sure that there are no bubbles in the line.
7. Exit the calibration program by using the LoggerPro menu in the main menu bar... “**Quit LoggerPro**”

C. To set up a titration using Vernier LabPro:

1. Open the “LoggerPro” program on the dock and then open the “LoggerProExperiments” folder on the desktop. Double click on the icon “**acidbasetitration.cmb1**”. The “acidbasetitration.cmb1” titration window will be displayed. As with the calibration software, the table on the left will record collected data in columns labeled with color-coded headings. A small window under the table will display pH (live reading). Temperature will be displayed if the temperature probe is connected to the interface. The larger graphing window will display your data as it is being recorded by the interface.

The “X” axis is the volume (μL) delivered by the pump and is set from 0 to 20,000 μL . The “Y” axis is the sensor output and is set to record pH 0 to 12. (NOTE: If necessary, the axes may be rescaled at anytime by clicking on the minimum/maximum value and typing in the preferred value.) “**Clear data**” under the “**Data Menu**” in the Logger Pro Menu bar to erase stored data and clear the window.

- To calibrate the volume (“X”) axis, use the scroll bar at the bottom of the table, to find the volume column and double click on the heading. A “**Calculated Column**” window will appear. Find the equation box and change ONLY the last number in the equation to the calibrated pulse volume in μL that your pump delivers. Click “**Done**”.
- To calibrate the pH electrode chose the “**Experiment**” menu from the LoggerPro menu bar and choose “**Calibrate**”. Sliding to the right choose “**LabPro: 1CH1: Electrode Amplifier**”. The sensor settings window will appear. Make sure that the current calibration is set for “Electrode Amp pH<Sensor Page 1>”. Click on the “**calibrate now**” button. This is a two-point calibration. Remove the pH electrode from the electrode storage solution, make sure that the hole in the blue collar is open, rinse well with deionized water and place in the pH 4.00 (pink) buffer solution. Always make sure the ground glass frit is covered by solution and no bubbles are caught in the protective cage. Once the “volts” reading has stabilized, type “4.00” into the “**enter value**” box and press the “**Keep**” button. Repeat to calibrate in pH 10.00 buffer solution and press the “**Keep**” button. Press “**Done**”.
- Add 50 mL of deionized water to a clean, dry 100-mL beaker (lines on beaker are OK). Using a graduated cylinder, add 10 mL of amino acid to the water, place the beaker on the stir plate, and add a stir bar. Rinse and dry the pH electrode and carefully place it in the beaker. Rest the electrode in the extended portion of the lip of the beaker. Carefully turn the stir plate on and arrange the beaker so that the stir bar does not hit the electrode cage. Adjust the pH to ~ 1.5 with 1.0 M HCl. Using the provided clip, attach the pump outlet tube to the beaker so that the end of the tubing is right at the level of the solution being tested.
- Press the green “**Collect**” button in the main window menu bar to start the titration. For the first titration, click “**erase and continue**”. The data will be recorded in the table and the titration and derivative plots will automatically be displayed on the graph.
 - NOTE: If this is not true, double click on the “**Sensor Output**” “Y” axis label and choose “**More**”. Then check the pH and dpH/dV boxes for the appropriate run. Remove the check for any unwanted data being displayed on the graph.
- Press “**Stop**” when the pH has reached ~ 12 -13 to end the titration (“Stop” is where the “**Collect**” button was at the start of the run). The color of the curves will match the color of the headings to the columns in the table. Choose “**File**” from the **Logger Pro** Main window menu bar and “**Save**” to assure that the data is not lost while preparing the next.
- When all of the titration experiments are completed, and have been saved, the graph can be printed by choosing “**File**” from the **LoggerPro** main window menu bar and “**Print**”

Graph". Entering the information in the "**Printing Options**" window will provide a label to the hardcopy of your graph, if the "**print footer**" box is checked.

8. To analyze the data points of interest on the pH and derivative plots, press the "**X=**" (also called the "**Examine**") button in the LoggerPro titration window menu bar. The color of the data values will match the color of the plotted data. Any point along the curves can be evaluated (also recorded in the table). Record the data points that are important to your experiment. Click on the "**X=**" button again to exit data analysis.
9. You can also press the "**Control-Option**" keys on the keyboard while holding down the mouse with the cursor placed on the graph. Choosing "**copy**" from the menu box will allow the graph to be pasted into a Word document. Open Word and use "**Paste**" under the "**Edit**" menu to copy the graph. The graph can be further edited in Word. If you use the "**Save as**" option to save the data, you would need the LoggerPro Software on your computer to reopen the file.
10. To exit the program, choose "**Quit LoggerPro**" from the **LoggerPro** menu bar. Press "**Save**".
 - NOTE: When clearing data and/or erasing and continuing between titrations, always check that the pump calibration volume (pulse volume) has not been changed to the default value (it should be set the volume that you determined previously).

NOTE: IF YOU ARE FINISHED WITH THE VERNIER SYSTEM, THE TITRANT MUST BE FLUSHED FROM THE PUMP:

- Reopen the "**one minute pump calibration.cmb1**" and place the inlet tubing into a beaker of deionized water. Place the outlet tubing in a waste beaker. Be sure that the line is secure and that the open end of the tubing is placed at the bottom of the beaker to reduce bubbles in the line. Use at least 90 pulses to run deionized water through the pump and tubing. Then place the inlet hose on the bench top and use 60 pulses to dry the lines.
- Please rinse the pH electrode and place it in the electrode storage solution (the hole in the blue collar should be open).
- Unplug the system (both plugs) when done.