Ionization Constants of Cysteine

Introduction

Cysteine is an important amino acid which, when protonated at low pH, has three acidic hydrogens: carbonyl (—COOH), amino (—NH$_3^+$), and thiol (—SH):

\[
\begin{align*}
 & H \\
 & HS \quad CH_2 \quad C \quad COOH \\
 & NH_3^+
\end{align*}
\]

This experiment measures the equilibrium constants (acid ionization constants) for these three protons through a combination of an acid-base titration and spectrophotometric measurements.

This experiment is very similar to the one described by G. E. Clement and T. P. Hartz, *J. Chem. Educ.* **48**, 395 (1971). A more recent reference on this subject (A. G. Splittgerber and L. L. Chinander, *J. Chem. Educ.* **65**, 167 (1988)) introduced two changes to the procedure described by Clement and Hartz. Their borax buffers are not very stable, so they have been replaced with ammonium chloride buffers to make up solutions at four intermediate pHs. Also, spectrophotometric measurements will be made over a range of wavelengths, not just at 232 nm as Clement and Hartz suggested.

You should be familiar with both papers (each only three pages long) before starting the experiment. Links to them are available on the course web site for this experiment. Note that you must access the journal sites for these papers from a dartmouth.edu Internet address; access is controlled via license to the Dartmouth library.

Experimental

(1) **Potentiometric Acid-Base Titration:** Accurately weigh about 1.2 g of cysteine hydrochloride monohydrate, C$_3$H$_7$NO$_2$S·HCl·H$_2$O (M.W. 175.62), and dissolve it to the mark in a 50 mL volumetric flask using distilled water through which N$_2$ has been bubbled for a few minutes to reduce its CO$_2$ and O$_2$ content. After standardizing the pH meter with the provided buffer solutions, pipet 25.0 mL of the cysteine hydrochloride solution, and titrate it with the provided 0.500 M NaOH solution under a nitrogen atmosphere. Record the pH of the solution after each 0.25 mL addition of titrant solution.

(2) **Spectrophotometric Determination of Sulfhydryl Ionization:** Stock solutions of 0.005 M acetate buffer (pH ≈ 4.5) and a 0.015 M sodium hydroxide solution are available as low and high pH media. Four different stock ammonium chloride buffer solutions, each with different pH (in the range 10–11.5) will also be provided. Make a
fresh stock solution of cysteine hydrochloride, about $4.0 \times 10^{-3}$ M (~31.5 mg), in a 50 mL volumetric flask. Pipet six 1.0 mL aliquot portions of this solution into six 25 mL volumetric flasks and dilute each with a different buffer (one acetate buffer, four ammonium chloride buffers, and one with the sodium hydroxide solution) to end up with 6 solutions of different pH. Measure the pH of each of the six solutions, and then measure the absorbance spectrum in a 1 cm path length cell over the range 220–255 nm. For each solution, record the absorbance readings at 232 and 248 nm. From the absorbance values at 232 nm, calculate the corresponding values of the fractional sulfhydryl ionization by means of Equation (6) in Clement and Hartz. Repeat your calculations using the values at 248 nm.

Calculations

A computer program entitled “Cysteine Analysis” is available in the lab (see your TA) to determine values (and associated uncertainties) for $pK_1$, $pK_2$ and $pK_3$ that give the best fit of your data to the theoretical titration curve. To implement the program, trial values of the ionization constants are needed. There is a simple and systematic way of arriving at these. First plot (using the Cysteine Analysis program) the experimental pH curve against titrant volume. A sharp rise in pH will be noted at a titration volume of around 7 to 8 mL. The inflection point at that sharp rise corresponds to the equivalence point for the titration of the carboxyl group. Compare the number of moles of base added at that point to the number of moles of cysteine weighed; agreement should be very good. If the discrepancy is more than 1%, either the cysteine weight or the base concentration is in error or your titration technique is faulty. Assume that the cysteine weight was in error and adjust the acid concentration to obtain agreement at the equivalence point. No amount of fiddling with the K’s can compensate for faulty stoichiometry.

Now recall that for any monoprotic acid, the pH at the half-equivalence point is very simply related to $pK$. For any Brønsted acid-base pair, HA and $A^-$:

$$HA \rightleftharpoons A^- + H^+ \quad K = \frac{[H^+][A^-]}{[HA]}$$

Thus at the half-equivalence point, when $[A^-]$ is equal to $[HA]$, the $H^+$ molarity is equal to $K$. One can see the same point from the so-called Henderson-Hasselbach equation. Taking negative logs of the above equation and rearranging gives:

$$pH = pK + \log_{10} \left( \frac{[A^-]}{[HA]} \right)$$

Thus, when $[A^-]$ equals $[HA]$ the solution pH is equal to the pK of the HA species. For an acid with a single ionizable hydrogen, the HA and the $A^-$ species do have equal
concentrations at the half equivalence volume, and the pH at this point should be an excellent estimate of pK.

In polyprotic acids for which successive pKs differ sharply (5 units or so), the various classes of protons are titrated essentially independently and the above ideas again apply almost exactly. That is, the pH at one half the equivalence volume for titration of the first hydrogen is pK$_1$, the pH at three-halves of the first equivalence volume is a good estimate for pK$_2$, and so on.

More commonly, the pK values for polyprotic acids are not well enough separated for the above recipes to apply exactly, since more than one equilibrium reaction must be considered at each point during the titration, i.e., there will be several Brønsted acid-base pairs present at appreciable concentrations simultaneously. However, the above ideas still retain some validity at any point in the titration where one conjugate pair predominates. In the cysteine case, even though pK$_2$ and pK$_3$ are not well separated, good initial estimates of their values may be obtained from your titration curve by simply reading the pH at three-halves and five-halves, respectively, of the initial carboxylic acid equivalence volume. An excellent estimate of pK$_1$ is available as the pH of the half equivalence volume.

Having found K$_1$, K$_2$, and K$_3$ as directed above, evaluate the "microscopic" ionization constants k$_s$, k$_n$, k$_ns$, and k$_sn$ from Equations (3), (4), and (7) of Clement and Hartz, and report these as pK values along with pK$_1$, pK$_2$, and pK$_3$. It is easiest first to get k$_s$ from Equation (7) and your values of $\alpha_s$, choosing a suitable average value from the results. Then get k$_n$ from Equation (3) and the remaining two microscopic constants from Equation (4).
Questions

1. Derive Equations (3), (4), and (7) in the article of Clement and Hartz from the ionization scheme of Equations (1) and (2) and the treatment of sulfhydryl ionization embodied in Equations (5) and (6).

2. Calculate the percentage of sulfhydryl groups ionized in cysteine at the physiological pH of 7.4.

3. From reading the paper of Splittgerber and Chinander, you should realize that the concern with the old procedure had to do with a wavelength dependence of the absorbances, which affected the validity of an important approximation used in the calculations. What is this approximation, and when is it believed to be valid?

4. **OPTIONAL (for up to 10% extra credit)** Table 1 compiles literature values of cysteine ionization constants in a more complete set than in the table of Clement and Hartz. Note that the ionic strength I affects the apparent equilibrium constants; this is particularly noticeable at I = 1.0, where all pK values are appreciable smaller than at lower I. Qualitatively, this apparent increase in acidity may be understood as follows.

Consider again the dissociation of weak acid HA:

\[ HA \rightleftharpoons A^- + H^+ \]

\[ K = \frac{[H^+][A^-]}{[HA]} \]

(Note: Recall that the thermodynamic ionization constant, which will be called \( K_a \) here, is written with activities rather than concentrations.) When the dissociation takes place in a solution of finite ion concentrations, whatever the chemical identity of those ions, the \( H^+ \) and \( A^- \) ions will accumulate about themselves a diffuse ion atmosphere of net opposite sign. The attractive interactions between, say, an \( A^- \) ion and its net positively charged ion atmosphere represent a lowering of the free energy of the system (compared to a zero ionic strength situation) and thus to a shift in the equilibrium toward the right. Thus at finite ionic strength, \( K \) is larger than at zero ionic strength and pK is smaller. (The thermodynamic constant \( K_a \) will, of course, not be changed, since the free energy changes will appear in the activity coefficients.)

This effect is described quantitatively for a monoprotic acid by the following equation:

\[ K_a = \gamma_\pm^2 \frac{[H^+][A^-]}{[HA]} \]

where \( \gamma_\pm \) is the mean ionic activity coefficient and where we ignore the distinction between molality and molarity for the low concentrations used in this experiment. For a 1:1 electrolyte, \( \gamma_\pm^2 = \gamma_+\gamma_- \) and when this is substituted into the expression for \( K_a \), we find
\[
K_a = \gamma_+ [H^+] \gamma_- \left[ \frac{A^-}{[HA]} \right] = \alpha_{H^+} \gamma_- \left[ \frac{A^-}{[HA]} \right]
\]

where the activity \( \alpha_{H^+} \) has been written for \( \gamma_+ [H^+] \) in the second equality. Since the pH meter responds to \( \alpha_{H^+} \) rather than \( [H^+] \), the apparent ionization constant obtained in this experiment is

\[
K_{exp} = \alpha_{H^+} \left[ \frac{A^-}{[HA]} \right]
\]

so that\(^1\)

\[
K_a = \gamma_- K_{exp}
\]

Since the activity coefficients are usually less than unity, \( K_a \) will be smaller than \( K_{exp} \), where we recall that \( K_a \) is \( K_{a2} \) (second ionization), in agreement with the qualitative discussion.

The magnitude of \( \gamma_- \) may be estimated by use of the Debye-Hückel limiting law in the form

\[
\ln \gamma_- = -z_-^2 C \sqrt{\frac{\gamma I}{1 + a B \sqrt{I}}}
\]

where \( z_- \) is the charge, \( B \) and \( C \) are constants: \( C = 1.174 \) (kg/mol)\(^{1/2} \), \( B = 3.284 \times 10^9 \) (kg/mol)\(^{1/2} \) m\(^{-1} \), the ion size \( a \) may be taken to be \( 3 \times 10^{-10} \) m, and \( I \) is the ionic strength in mol/kg units formally, i.e., molality, but it is a good approximation to equate molarity and molality here.

So here’s the question, finally: Estimate \( I \) for one of your spectrophotometric experiments at pH > 7, and hence estimate the approximate correction \( pK_a - pK_{exp} \) that should be applied to the experimental \( pK_{exp} \).

\(^1\) Note that these equations are valid only when the dissociating monoprotic acid is a neutral species, as in the second ionization of cysteine. For the first ionization, where the acid carries a single proton charge, \( \gamma_+^2 \) is replaced by \( \gamma_+ \) (for \( H^+ \)/\( \gamma_+ \) (for \( HA^+ \)) and we have \( K_{a1} = K_a \) (first ionization) = \( K_{exp} / \gamma_+ \) (for \( HA^+ \)).
TABLE 1

Literature Values for Cysteine Dissociation Constants

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