Assigning the pK_a's of Polyprotic Acids

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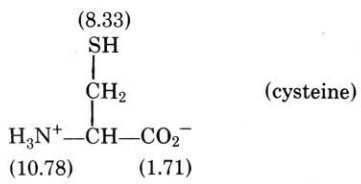
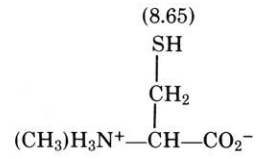
While teaching a biochemistry lab course in which students measured the pK_a's of amino acids, the author was asked about the assignment of the pK_a's of cysteine, which was reported in the textbook as follows.



(cysteine)

He blithely answered that this assignment made sense, and picked up a copy of the "Handbook of Biochemistry" [1] to look up data that might prove its validity to the student. He started by noting that H_2S (7.00) was more acidic than the NH_4^+ ion (9.24) and that the difference between the pK_a's of H_2S and NH_4^+ (2.24) was roughly equivalent to the difference between the second and third pK_a's of cysteine (2.45).

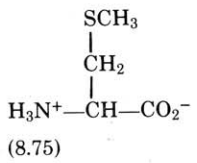
He then naively suggested that the simplest way to test this assignment would be to see which pK_a disappeared when either the $-NH_3^+$ or -SH group of cysteine was derivatized. He was therefore quite pleased to find an entry for N,N,N-trimethyl cysteine,



(N,N,N-trimethyl cysteine)

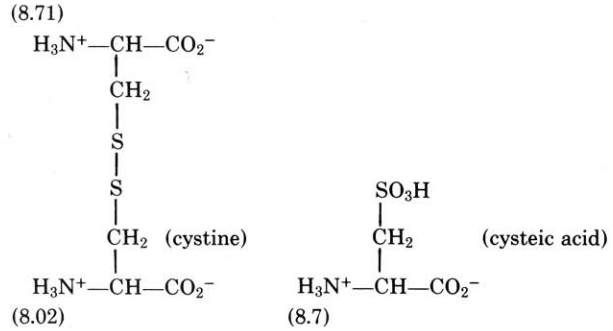
which showed the disappearance of the pK_a at 10.78, in accord with the textbook assignment.

Unfortunately, the student found an entry for S-methyl cysteine which showed that derivatization of the sulfhydryl group also leads to the disappearance of the pK_a at 10.78.



(S-methyl cysteine)

When we then turned to "Dissociation Constants of Organic Bases in Aqueous Solution" [2], the student noted that the pK_a data for both cysteine and cysteic acid were also in agreement with an assignment of pK_2 and pK_3 for cysteine that is the opposite of what appears in most textbooks.



Polyprotic Acids for which the Difference between K_a's is Large

The answer to why derivatization of cysteine at either the $-NH_3^+$ or -SH groups leads to the loss of pK_3 is well known to biochemists but not as well known to chemists who are used to working with polyprotic acids such as H_2S, H_2CO_3, and H_3PO_4, or simple amino acids such as glycine.

Classical treatments of polyprotic acids are based on the assumption that the difference between the pK_a's is large enough to assume stepwise dissociation.

$$H_nM + H_2O \rightleftharpoons H_{n-1}M^- + H_3O^+ \qquad K_1$$

$$H_{n-1}M^- + H_2O \rightleftharpoons H_{n-2}M^{2-} + H_3O^+ \qquad K_2, \text{ etc.}$$

If the dissociation is in fact stepwise, most of the H_3O^+ ion comes from the loss of the first proton, and the equilibrium concentrations of the H_3O^+ and $H_{n-1}M^-$ ions are more or less equal.

$$[H_3O^+] \simeq [H_{n-1}M^-]$$

Substituting this approximation into the equilibrium expression for the *first step* in the dissociation allows us to solve for the H_3O^+ or $H_{n-1}M^-$ ion concentration.

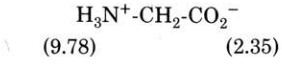
$$[H_3O^+] \simeq [H_{n-1}M^-] = \sqrt{K_1[H_nM]}$$

Substituting the same approximation into the expression for the *second step* suggests that the concentration of the $H_{n-2}M^{2-}$ ion is roughly equal to the value of K_2 for the acid.

$$\frac{[H_3O^+][H_{n-2}M^{2-}]}{[H_{n-1}M^-]} = K_2$$

The Henderson-Hasselbach Equation

When there is a large difference between the pK_a's of an asymmetric polyprotic acid such as glycine, we can assign these pK_a's to individual titratable groups. For example,



We can then use the Henderson-Hasselbach equation,

$$pH = pK_a + \log \frac{[base]}{[acid]}$$

to calculate the percentage of each titratable group in its acid or conjugate base form at a given pH. At a pH of 7.00, for example, only 1 in 600 of the amino groups in glycine is present as the conjugate base ($-NH_2$), and only 1 in 45,000 of the α-carboxylic acid groups is present as the conjugate acid ($-CO_2H$).

Polyprotic Acids for which the Difference between K_a's is Small

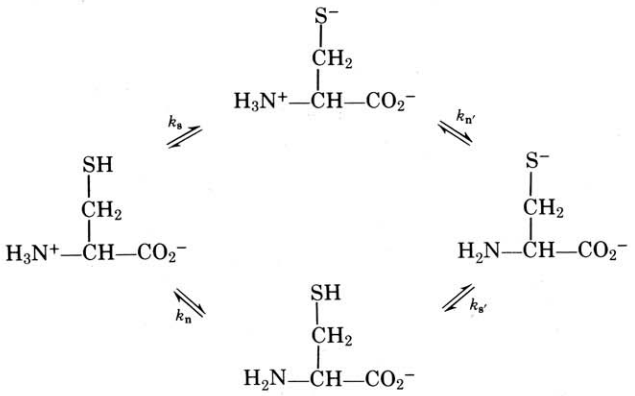
Cysteine is a classic example of a polyprotic acid for which

[1] Sober, H. A., Ed., "Handbook of Biochemistry", 2nd ed.; Chemical Rubber Company: Cleveland, OH, 1970.

[2] Perrin, D. D. "Dissociation Constants of Organic Bases in Aqueous Solution"; Butterworths: London, 1965.

the stepwise dissociation assumption fails.[3] When cysteine is titrated with base, a significant fraction of the -SH groups are deprotonated at more or less the same time as the $-NH_3^+$ ions.

Because the presence or absence of a charge on the -SH group can influence the acidity of the $-NH_3^+$ group, and vice versa, it is possible to define four *microscopic dissociation constants* for cysteine (k_n, k_s, $k_{n'}$ and $k_{s'}$) that describe what happens when the -SH and $-NH_3^+$ groups are titrated.



These *microscopic* dissociation constants are associated with the following equilibria.

$$k_s = \frac{[H_3O^+][H_3N^+\text{--}S^-]}{[H_3N^+\text{--}SH]}$$

$$k_n = \frac{[H_3O^+][H_2N\text{--}SH]}{[H_3N^+\text{--}SH]}$$

$$k_{s'} = \frac{[H_3O^+][H_2N\text{--}S^-]}{[H_2N\text{--}SH]}$$

$$k_{n'} = \frac{[H_3O^+][H_2N\text{--}S^-]}{[H_3N^+\text{--}S^-]}$$

Because two products are formed when the $H_3N^+CH(CH_2SH)CO_2^-$ ion loses a proton, H_2N--SH and the H_3N^+--S^- ion, the equilibrium concentration of the conjugate base in this dissociation is equal to the sum of the concentrations of these products. The equilibrium expression for the *macroscopic* K_2 constant which measures the ease with which cysteine loses a second proton is therefore,

$$K_2 = \frac{[H_3O^+]([H_3N^+\text{---}S^-] + [H_2N\text{---}SH])}{[H_3N^+\text{--}SH]}$$

Both the H_2N---SH and H_3N^+---S^- produced in the second dissociation of cysteine can go on to lose a third proton to form the H_2N---S^- ion. Because two acids, H_2N---SH and H_3N^+---S^-, serve as sources of the H_2N---S^- conjugate base, the equilibrium expression for the *macroscopic* K_3 dissociation constant for cysteine is

$$K_3 = \frac{[H_3O^+][H_2N\text{--}S^-]}{[H_3N^+\text{--}S^-] + [H_2N\text{--}SH]}$$

What is the relationship between the *macroscopic* (K_2 and K_3) dissociation constants measured when cysteine is titrated and the *microscopic* or *molecular* (k_s, k_n, etc.) dissociation constants of the $-NH_3^+$ and -SH groups? By juggling the equilibrium expressions for K_2, K_3, k_s, k_n, $k_{s'}$, and $k_{n'}$ we can see that,

$$K_2 = \frac{[H_3O^+]([H_3N^+\text{--}S^-] + [H_2N\text{--}SH])}{[H_3N^+\text{--}SH]} = k_n + k_s$$

$$\frac{1}{K_3} = \frac{[H_3N^+\text{--}S^-] + [H_2N\text{--}SH]}{[H_3O^+][H_2N\text{--}S^-]} = \frac{1}{k_{s'}} + \frac{1}{k_{n'}}$$

and,

$$k_s k_{n'} = k_{s'} k_n = \frac{[H_3O^+]^2[H_2N\text{--}S^-]}{[H_3N^+\text{--}SH]} = K_2 K_3$$

The *macroscopic* dissociation constants (K_2 and K_3) measured when cysteine is titrated are therefore related to the acidity of both the $-NH_3^+$ and -SH groups, and it is a mistake to attempt to assign these *macroscopic* constants to either the -SH or $-NH_3^+$ group of cysteine unless there is reason to believe that there is a significant difference in the magnitude of the *microscopic* constants k_s and k_n.

Analysis of Microscopic Dissociation Constants for Cysteine

An analysis of UV spectral data for cysteine derivatives enabled Benesch and Benesch[4] to determine the *microscopic* pk_a constants for cysteine.

$$pk_s = 8.53$$

$$pk_n = 8.86$$

$$pk_{s'} = 10.03$$

$$pk_{n'} = 10.36$$

These data suggest that the *microscopic* or *molecular* dissociation constants for the -SH and $-NH_3^+$ groups in cysteine are virtually identical. Furthermore, the loss of a proton by either the -SH or $-NH_3^+$ groups leads to a decrease of about 1.5 pK units in the acidity of the other functional group, as might be expected.

Analysis of pK_a Data

The close similarity between the *microscopic* dissociation constants for the $-NH_3^+$ and -SH groups in cysteine might appear surprising at first glance, since the pK_a's of H_2S and NH_4^+ differ by 2 pH units. However, the pK_a of a more reasonable model compound, CH_3CH_2SH (10.60), is remarkably close to the pK_a of $CH_3NH_3^+$ (10.66), and the results of this analysis of cysteine are therefore not particularly surprising.

Conclusion

Because the *microscopic* pk_a's of the -SH and $-NH_3^+$ groups in cysteine are almost the same, derivatization of either group leads to the disappearance of the same *macroscopic* pK. The difference of roughly 2 pK units between pK_2 and pK_3 of cysteine is not a reflection of the relative acidities of the -SH and $-NH_3^+$ groups but the effect of the loss of a proton by either the -SH or $-NH_3^+$ group on the acidity of the remaining functional group.

Although it is tempting to assign the pK_a's of polyprotic acids to individual titratable groups, this exercise should be reserved for those acids where the difference between the K_a's is large enough that we can safely assume stepwise dissociation.

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[3] Edsall, J. T.; Wyman, J. "Biophysical Chemistry"; Academic: New York, 1958; Vol 1 pp 450–504.

[4] Benesch, R. E.; Benesch, R. *J. Amer. Chem. Soc.* **1955,** *77,* 5877.