

Methods in Enzymology

Volume XI

Enzyme Structure

EDITED BY

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[84] Examination of Titration Behavior

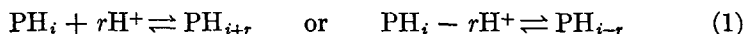
By YASUHIKO NOZAKI and CHARLES TANFORD

I. Introduction

It is believed that all enzymes belong to the class of globular proteins. Not a single case to the contrary is known. The chemical structure of such protein molecules is, as far as titration is concerned, a number of dissociable side-chain groups attached to a peptide chain, in addition to an amino group residing at one end and a carboxyl group at the other end of the chain. The titration behavior of a protein therefore will be similar to that of a mixture of the constituent amino acids with their α -amino and α -carboxyl groups blocked, except for the terminal amino acids.

The information obtained from titration curves of enzymes based on this fact is therefore of an analytical nature. There is, however, an important difference between the titration of a protein and the corresponding mixture of blocked amino acids. Deviation of the former from the latter tells us the nature and magnitude of interactions affecting the titration; therefore, information about the conformation of protein molecules is obtained. A comprehensive review of the subject has recently been published,¹ to which the reader is referred for detailed information to supplement the brief remarks in this section.

Electrometric Titration Curve. An electrometric (direct) titration curve, strictly speaking an electrometric hydrogen-ion titration curve, represents the relation between pH and the number of moles of protons bound by, or removed from, 1 mole of protein in the reaction,



where PH_i represents a protein species at the beginning of the titration, and r is the number of hydrogen ions bound by, or removed from, the protein. Experimental r values are obtained as the difference between the number of moles of strong acid or base added to a solution containing 1 mole of protein to bring its pH from initial to final values, and the number of moles of strong acid or base added to the solvent only to bring about the same pH change with other conditions (ionic strength, volume, etc.) being the same as for the protein solution. In practice, the number of moles of free hydrogen or hydroxyl ions is calculated from the pH values measured in the presence of protein, rather than being determined

¹ C. Tanford, *Advan. Protein Chem.* **17**, 69 (1962).

each time from measurements on a control solution without protein. The procedure is based on the following assumptions:

(1) Hydrogen ion activity can be known with sufficient accuracy from pH measurement,² with the aid of the assumption of single ion activity.³⁻⁵ It means that we can identify the experimental pH with the negative logarithm of hydrogen ion activity, a_H , which in turn can be considered the product of hydrogen ion concentration, C_H , and an activity coefficient, γ'_H . Thus,

$$pH = pa_H = pC_H + p\gamma'_H \quad (2)$$

where p in each symbol stands for the negative logarithm.

(2) We maintain the idea of a constant ion product, K_w , for the dissociation of water, and extend the idea of single ion activity to hydroxyl ions, so that we may write:

$$pOH = pK_w - pH = pC_{OH} + p\gamma'_{OH} \quad (3)$$

A prime mark accompanies both activity coefficients to indicate that they are apparent activity coefficients, since the pH as defined by the experimental measurement, and therefore pOH also, is somewhat different from pa_H or pa_{OH} defined by more rigorous procedures.³

(3) The apparent activity coefficients remain the same, at constant ionic strength, in the presence and absence of protein in the solution.

In the calculation of ionic strength we take into account the concentrations of added salt and acid or base, ignoring the possibility that some of the added ions are bound by the protein. It is found that $p\gamma'_H$ and $p\gamma'_{OH}$ values are not affected very much by the equipment used, including the kind of glass electrode, nor by technique, such as stirring or static titration. They remain constant within a narrow range to very low and high pH. Typical values of $p\gamma'_H$ and $p\gamma'_{OH}$ for KCl solutions are listed in Table I.

Apparent activity coefficients obtained by titrating guanidine hydrochloride solutions are also known to depend only on the concentration of guanidine hydrochloride (GuCl) but not on pH.⁶ The values at several GuCl concentrations are listed in Table II for comparison with those values listed in Table I.

The number of moles of bound hydrogen ions per mole of protein,

² C. Tanford, in "Electrochemistry in Biology and Medicine" (T. Shedlovsky, ed.), p. 251. Wiley, New York, 1955.

³ R. G. Bates, "Electrometric pH Determinations," Wiley, New York, 1954.

⁴ R. G. Bates and E. A. Guggenheim, *Pure Appl. Chem.* **1**, 166 (1960).

⁵ H. S. Frank, *J. Phys. Chem.* **67**, 1554 (1963).

⁶ Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.* **89**, 736 (1967).

TABLE I
APPARENT ACTIVITY COEFFICIENTS OF HYDROGEN AND HYDROXYL
IONS IN KCl SOLUTIONS AT 25°

Ionic strength (KCl-HCl or KCl-KOH)	$p\gamma'_{\text{H}}$	$p\gamma'_{\text{OH}}$
0.01	0.054	0.056
0.1	0.076 ^a	0.116
0.15	0.073	0.143
0.25	0.074	0.143
1.0	0.022	0.162

^a The pH value assigned to 0.01 M HCl-0.09 M KCl by Bates is 2.073; this would give a $p\gamma'_{\text{H}}$ value of 0.073 [R. G. Bates, *J. Res. Natl. Bur. Std.* **45**, 418 (1950)].

calculated with the aid of such activity coefficients, is plotted against pH. Typical titration curves are shown in Fig. 1. The $\bar{r}$ scale shows the number of moles of hydrogen ions dissociated from 1 mole of protein in its most acidic form, i.e., where all dissociable groups are present in the acidic form, and the protein molecule carries the maximum possible number of bound hydrogen ions.

Starting from the zero point on the $\bar{r}$ scale (i.e., the point of maximum net proton charge), as an amount of alkali is added the first groups which lose their hydrogen ions are those with lowest pK , presumably

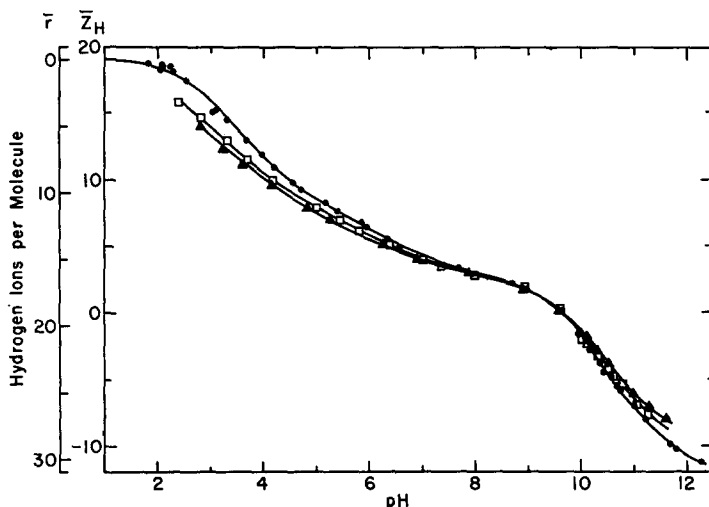


FIG. 1. Titration curves of ribonuclease in KCl solutions at 25° [C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.* **78**, 5287 (1956)]. Ionic strengths are 0.15 (●), 0.03 (□), and 0.01 (▲).

TABLE II
APPARENT ACTIVITY COEFFICIENTS OF HYDROGEN AND HYDROXYL IONS
IN GuCl SOLUTIONS AT 25°

GuCl concentration (mole/liter)	$p\gamma'_H$	$p\gamma'_{OH}$
4.0	(-0.34)	—
4.5	(-0.41)	—
5.0	-0.495	0.975 ^a
5.5	-0.592	1.011 ^a
6.0	-0.686	1.046 ^a

^a The large positive values reflect hydrolysis of GuCl with formation of the free base.

carboxyl groups,⁷ and the net proton charge on the protein molecule decreases as a result of the negative charges introduced thereby. As more alkali is added and the pH rises, the more basic groups, among which the imidazole groups of histidine will usually be the first, will lose their hydrogen ions and their positive charges. Both effects are to reduce the number of proton charges and somewhere along the curve there will be a pH at which the protein molecules carry, on the average, zero net proton charge. This is the zero point on the $\bar{Z}_H$ scale. It is noted that the number of moles of protons bound from this point to the point of maximum net proton charge ($\bar{r} = 0$) is equal to the number of all basic groups (maximum acid-binding capacity, ΣN^+) on the protein molecule. The $\bar{Z}_H$ scale shows the number of protons bound by or removed from 1 mole of protein originally at the point of zero net proton charge. The pH at zero net proton charge ($\bar{Z}_H = 0$) is ordinarily close to the isoionic point of the protein.

Isoionic Point. The definition of the isoionic point adopted in this chapter is an operational one as follows: the pH of a solution which contains solvent water and the protein, free from ions other than hydrogen and hydroxyl ions. Such a solution can be prepared easily by the method described in Section IV below. The isoionic point is not a unique function but may depend on the protein concentration. $\bar{Z}_H$ at the isoionic point is usually close to zero, but may differ from zero if the pH is far from neutrality. Deviation from zero in that case simply reflects the fact that the protein has to acquire a net charge in order to compensate the charges of opposite sign due to excess H^+ or OH^- ions. In any event, $\bar{Z}_H$ at the isoionic point can always be calculated from the relation

$$\bar{Z}_H = (C_{OH} - C_H)/C_P \quad (4)$$

where C_{OH} , C_H , and C_P are the concentrations of free hydroxyl ions, hy-

⁷ Dissociating groups such as, say, carboxyl or imidazole cannot be positively identified without supporting evidence. See the argument in footnote 1 (p. 71).

drogen ions, and protein, respectively. (We may set $\text{pH} = -\log C_{\text{H}}$, and $\text{pOH} = -\log C_{\text{OH}}$, if the total ion concentration is low enough to permit equating activity coefficient to unity.) The difference between the isoionic point and the pH where $\bar{Z}_{\text{H}}$ is actually zero can then be measured from the titration curve directly. An unequivocal reference point of $\bar{Z}_{\text{H}} = 0$ can thus always be defined.

In practice a neutral salt is added to an isoionic protein solution to make up the ionic strength. It usually displaces the isoionic point slightly in a manner described by Scatchard and Black.⁸ Whenever large pH displacement occurs, it is customary to seek its interpretation in ion binding. However, it should be added that Eq. (4) is valid even in the presence of added neutral salt, whether its ions are bound by the protein or not, and $\bar{Z}_{\text{H}}$ can be calculated by Eq. (4) at any ionic strength.

Spectrophotometric Titration of Tyrosine Groups. It is desirable to have methods that measure the titration of specific groups, in addition to the electrometric titration so far described, which gives the total number of groups titrating. Titration of tyrosine groups by the change in absorption spectrum due to the dissociation of the phenolic hydroxyl groups is the only such method of discriminating titration in common use.⁹ In Fig. 2 are shown the spectrophotometric titrations of tyrosine phenolic groups of ribonuclease carried out under different conditions.

Group Counting. It is assumed that pK's of titratable groups on protein molecules are not completely different from pK's of similar groups on small molecules in a similar environment, especially with respect to electrostatic effects of neighboring groups. Those effects and eventually the assignment of reasonable pK values to the titratable groups on proteins have been the subject of many investigations. Nevertheless, we are still in an awkward position as to the assignment of accurate "normal" pK values to various titratable groups on a protein molecule. There are two approaches to the problem. One is to examine the pK values of model compounds that contain the same titratable groups as proteins do, and then to correct them for statistical factors and interactions, in order to evaluate an unperturbed or intrinsic pK (pK^{int}) for each kind of group. The second approach is to average pK's extracted from protein titration curves, after correcting for electrostatic interactions, and to assume that deviations from "normal" behavior are eliminated by the averaging process (i.e., it is assumed that positive and negative deviations occur with equal frequency). A list of values obtained in both ways is given in Table III.¹⁰

⁸ G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.* **53**, 88 (1949).

⁹ For recent progress in other methods, see footnote 1 (p. 80).

¹⁰ Footnote 1, Table I (pp. 73-74), Table V (pp. 112-113). Readers are also referred to J. T. Edsall in "Proteins, Amino Acids and Peptides" (E. J. Cohn and J. T.

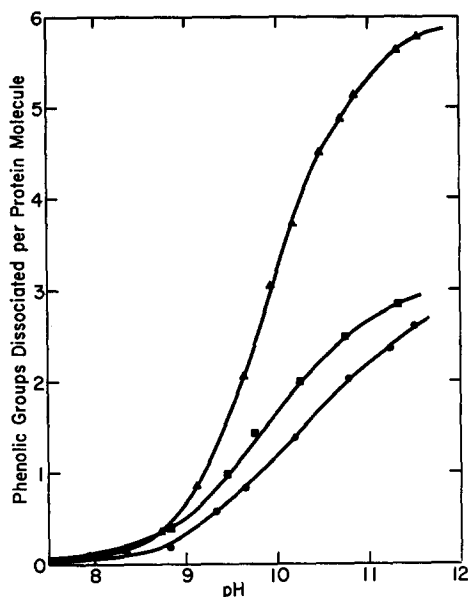


FIG. 2. Spectrophotometric titration of phenolic hydroxyl groups of ribonuclease at 25° [Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.* **89**, 736 (1967); also see C. Tanford, J. D. Hauenstein, and D. G. Rands, *J. Am. Chem. Soc.* **77**, 6409 (1955)]. Conditions: KCl, ionic strength, 0.01 (●); KCl, ionic strength, 1.0 (■); 6M GuCl (▲). Only the reversible portion of the data in KCl is shown.

It can be seen from Fig. 1 that the titration curve of a protein can usually be divided into three sigmoid regions, the transitions between them being at about pH 6 and pH 9. We may tentatively identify these regions as corresponding to the titration of (1) carboxyl groups, (2) imidazole plus α -amino groups, and (3) phenolic hydroxyl plus ϵ -amino groups. Thiol groups of cysteine residues, if they titrate, will be included in the last region. Guanidine groups of arginine are not titrated because of their high pK , although they are included in ΣN^+ .

The numbers of groups in each region of the titration curve can be obtained by direct counting. Because of overlap, the results are not very accurate but are useful for the first analysis of the curve, together with a knowledge of ΣN^+ if the isoionic point is known. We usually start from the acid end of the curve at, say, pH 2, where all carboxyl, thiol, and phenolic hydroxyl groups are uncharged, and all basic groups have

Edsall, eds.), Chap. 4, 5, 20. Reinhold, New York, 1943; see also J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Chap. 8, 9. Academic Press, New York, 1958.

TABLE III
INTRINSIC pK 's OF TITRATABLE GROUPS AT 25°

Type of group	Model compounds ^a	Proteins ^b	
		Dilute salt solution	GuCl 6 M
α -Carboxyl	3.8	3.6	3.1
Aspartyl carboxyl	4.0	(4.6) ^c	3.9
Glutamyl carboxyl	4.4	(4.6) ^c	4.35
Imidazole	6.3	6.6	6.4
α -Amino	7.5	^d	7.4
Thiol	9.5	^d	9.5
Phenolic	9.6	9.8	9.95
ϵ -Amino	10.4	10.0	10.3
Guanidyl	12	12	12

^a Obtained from pK values of small model compounds (see reference, footnote a, Table I). Corrections for electrostatic and other effects have been made to yield pK values representative of the unperturbed group.

^b Obtained from the titration of proteins, by application of Eq. (9). Values obtained for different proteins differ considerably, and the values listed represent somewhat arbitrary choices.

^c Protein titration curves in dilute salt solutions have heretofore been analyzed without regard for the anticipated difference between aspartyl and glutamyl COOH groups.

^d No reliable data have been obtained.

protonic charges. As stated previously, the first groups to titrate are carboxyls, up to about pH 6. The number should be the same as the total number of aspartic and glutamic acid residues of the protein molecule minus amide groups plus α -carboxyl groups. In the neutral region we find the number of histidine residues plus the number of α -amino groups. The number of groups titrated in the alkaline region is of least accuracy, because the alkaline end of the titration curve usually does not level off as the acid end does. The reason for this is that the titration of amino groups extends to well above the highest pH (pH 12) which can yield reliable data. However, the number of groups titrated in this region can be obtained with the aid of a short extrapolation. It should be equal to the sum of the numbers of tyrosine and lysine residues. The former can be obtained from spectrophotometric titration, and the latter can then be obtained as the difference. The transition from one region to the next is sharper at higher ionic strength for the same protein conformation, since the electrostatic interaction which makes the transitions less sharp diminishes as the ionic strength increases, as will be described below. Titration curves in concentrated guanidine hydrochloride show extremely sharp transitions, as will be seen in Section II below.

Of course, the actual groups titrated in any region is rarely exactly what it should be on the basis of the predictions just made, and therein lies the usefulness of titration curves as a tool in conformational analysis. Groups which do not titrate where expected must be in some special environment in the structure of the protein molecule.

Thermodynamic Interpretation. We shall confine ourselves to the very imprecise semi-empirical thermodynamic treatment in common use. A more sophisticated model has been presented elsewhere.¹¹ It should be emphasized that any thermodynamic treatment applies only to reversible portions of the titration curve, while group counting can be done on both reversible and irreversible portions, because it deals with chemical stoichiometry.

The heart of the approximate treatment is to assume that all groups with the same pK^{int} titrate simultaneously. Thus we may treat a titration curve as a combination of a small number of titration equilibria instead of having as many equilibria as there are titratable groups.

The nature of interactions between groups is considered to be solely electrostatic. A classical interpretation of this interaction is by the Linderström-Lang theory,¹² which is based on the following assumption: a protein molecule is represented by a sphere impenetrable to the solvent water and salt ions, and the charges on the molecule are smeared evenly over the surface of this sphere.

For dissociation of any one titratable group of type i , the equilibrium constant K_i is given by

$$K_i = a_H x_i / (1 - x_i) \quad \text{or} \quad pK_i = \text{pH} - \log x_i / (1 - x_i) \quad (5)$$

where x_i is the average degree of dissociation and a_H is the thermodynamic hydrogen ion activity. As a consequence of the protein model described above, the interaction encountered by a hydrogen ion which is being added to the protein molecule is dependent on the net charge only, and can be calculated as the work that must be done to increase the net charge by one unit, against the opposing force of the charges already present. The mode of dependence is found to be

$$K_i = K_i^{\text{int}} e^{2w\bar{Z}} \quad \text{or} \quad pK_i = pK_i^{\text{int}} - 0.868 w\bar{Z} \quad (6)$$

where K_i^{int} is the dissociation constant of titratable groups of type i in the absence of interaction, at $\bar{Z} = 0$ by assumption. The parameter w of Eq. (6) is given by

¹¹ C. Tanford and J. G. Kirkwood, *J. Am. Chem. Soc.* **79**, 5333 (1957).

¹² K. Linderström-Lang, *Compt. Rend. Trav. Lab. Carlsberg* **15**(7), 1 (1924); C. Tanford, "Physical Chemistry of Macromolecules," p. 537. Wiley, New York, 1961.

$$w = \frac{\epsilon^2}{2DkT} \left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a} \right) \quad (7)$$

where ϵ is the electronic charge, D is the dielectric constant of the medium, k is Boltzmann's constant, T is the temperature, b is the radius of the sphere which represents the protein molecule, a is the distance of closest approach of ions to the center of the sphere, i.e., the sum of the radii of the sphere and average small ions which make up the ionic strength, and κ is the Debye-Hückel parameter expressed by

$$\kappa = \left(\frac{8\pi N \epsilon^2}{1000 DkT} \right)^{1/2} I^{1/2} \quad (8)$$

where N is Avagadro's number and I is the ionic strength expressed by $I = \sum c_i z_i^2 / 2$, where c_i is the concentration of ion i as moles per 1000 ml and z_i is its charge. The value of w can be calculated from Eq. (7) with reasonable values for b and a , which are calculable either from the molecular weight and assumed hydration or from hydrodynamic data.¹³

It suffices here to point out that w depends on the ionic strength, has the same value for all titratable groups, and is constant as long as the protein molecule does not change its size and shape.

Combining Eqs. (5) and (6), we obtain

$$\text{pH} - \log x_i / (1 - x_i) = \text{p}K_i^{\text{int}} - 0.868 w \bar{Z} \quad (9)$$

It is assumed, at least initially, that chemically identical groups have the same $\text{p}K_i^{\text{int}}$ which, as was mentioned earlier, means that all groups of a given kind titrate to the same degree simultaneously at any pH. Thus Eq. (9) becomes applicable to a number of groups of the same type i instead of one group.

Equation (9) provides a way to evaluate $\text{p}K_i^{\text{int}}$ and w experimentally by plotting the left-hand side against $\bar{Z}$. As long as $\text{p}K_i^{\text{int}}$ and w remain the same, the plot will be a straight line and will give $\text{p}K_i^{\text{int}}$ as the intercept at $\bar{Z} = 0$, and $0.868 w$ as the slope. Such logarithmic plots are shown in Fig. 3. The w values thus obtained are often a little smaller than predicted from the spherical model by Eq. (7). This might be due to possible deviations of the actual shape from a sphere, as evidenced by the myoglobin molecule.¹⁴ On the other hand, when much too small w values have been obtained, as for α -corticotropin by Leonis and Li,¹⁵ one must assume a molecule entirely different from a sphere. In this case the molecule is probably a random coil. Sometimes a straight line is not ob-

¹³ Footnote 1 (p. 101).

¹⁴ J. C. Kendrew, H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips, and V. C. Shore, *Nature* **190**, 666 (1961).

¹⁵ J. Léonis and C. H. Li, *J. Am. Chem. Soc.* **81**, 415 (1959).

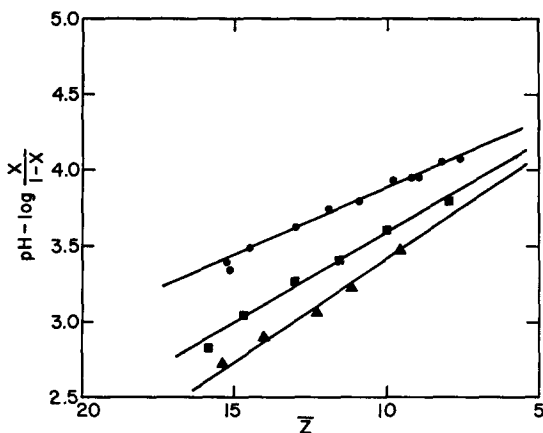


FIG. 3. Logarithmic plots of carboxyl titrations of ribonuclease at 25°. Data are from Fig. 1. Ionic strengths are 0.15 (●), 0.03 (■), and 0.01 (▲).

tained at all. This anomaly can mean heterogeneity among the groups being titrated, the wrong assumed number of groups, and/or configurational change in the pH range where curvature occurs. At any rate, one should not attempt to relate the w value to the actual conformation of the protein too strictly, since the spherical model itself is an oversimplification in the first place, but rather regard w as an empirical parameter.

II. Titration in Denaturing Media

Since almost all the titration curves of native proteins contain some anomalies, it is desirable to have a reference titration curve with which to compare the titration curve of the native protein, in order to pick out differences that point to involvement of titratable groups in the internal structure of the native molecule. The natural choice is the titration curve in a denaturing medium in which the protein is in the unfolded form, with virtually all titratable groups exposed to the solvent. The preferred denaturing agent is guanidine hydrochloride, because of its greater ability to unfold proteins than other reagents, such as urea, and because of its stability in solution (in contrast to urea, which is unstable¹⁶). In addition, guanidine hydrochloride is a strong electrolyte which should reduce electrostatic interactions almost to the vanishing point when concentrated solutions are used.

An example is provided by Fig. 4, which represents the titration of ribonuclease in 6 *M* guanidine hydrochloride. The results of the group

¹⁶ G. R. Stark, W. H. Stein and S. Moore, *J. Biol. Chem.* **235**, 3177 (1960).

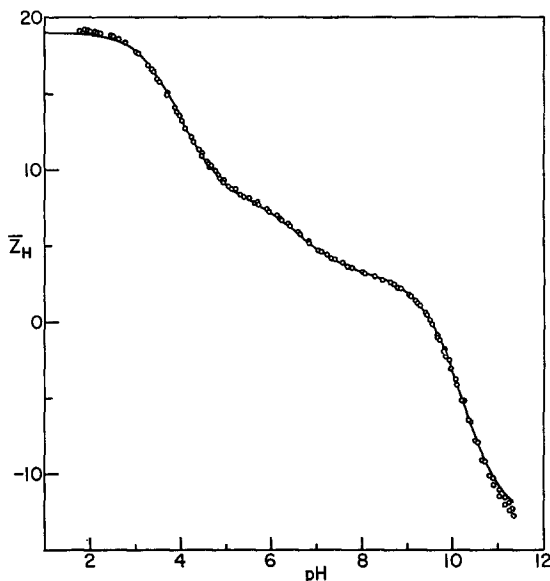


FIG. 4. Titration curve of ribonuclease in 6 M GuCl at 25° [Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.* **89**, 736 (1967)]. Solid line represents the theoretical curve which was constructed by using the following parameters: $w = 0$; 1 α -COOH, pK 3.4; 5 aspartyl COOH, pK 3.8; 5 glutamyl COOH, pK 4.3; 4 imidazole, pK 6.5; 1 α -NH₂, pK 7.6; 3 phenolic hydroxyl, pK 9.75; 3 phenolic hydroxyl, pK 10.15; 10 lysyl-NH₂, pK 10.35; 4 guanidiny, pK > 12.5.

counting procedure in this case are almost, but not quite, what is expected. The pH of zero net proton charge is 9.54, and ΣN^+ is close to 19 groups. This corresponds to the expected number (4 arginine, 10 lysine, 4 histidine plus an α -amino group). The number of groups titrated in the most acidic region is about 11, equal to the content of carboxyl groups (5 aspartic acid, 5 glutamic acid plus an α -carboxyl group). The number of groups titrated in the neutral region is 5, again as expected (4 histidine plus an α -amino group). The number of groups titrated in the alkaline branch of the curve, however, is more than expected on the basis of 10 lysine plus 6 tyrosine residues. This difference turned out to be due to the release of an extra proton in the β -elimination reaction of cystine residues, which leads to the eventual formation of lysinoalanine by a condensation with a neighboring lysine, as first reported by Patchornik and Sokolovsky¹⁷ and Bohak.¹⁸

¹⁷ M. Sokolovsky and A. Patchornik, *J. Am. Chem. Soc.* **86**, 1859 (1964); A. Patchornik and M. Sokolovsky, *ibid.* 1860 (1964).

¹⁸ Z. Bohak, *J. Biol. Chem.* **239**, 2878 (1964).

Preliminary experiments with other proteins in guanidine hydrochloride suggest that the same or similar reactions occur there also. The covalent structure of a protein (at least if it contains disulfide bonds) appears not to be stable at high pH, and reactions occur which result in the appearance of titratable groups. Apart from such anomalies at high pH, group counting in a medium such as 6 *M* guanidine hydrochloride should give the expected result for all proteins, i.e., it should be equivalent to a true analysis for the numbers of titratable groups on a protein molecule.

The spectrophotometric titration curve of the phenolic groups of ribonuclease in guanidine hydrochloride is shown in Fig. 2. It is seen that all six tyrosines are titrated together, as expected.

When the groups of any one kind, for instance the phenolic hydroxyls as shown above or side-chain carboxyls, are taken,¹⁹ and Eq. (9) is applied to their titration, values of w close to zero are obtained, as expected. The value of w is actually not quite equal to zero, if it is assumed that all groups of either kind titrate simultaneously. (For ribonuclease, $w = 0.021$ has been obtained from either phenolic hydroxyl groups or β,γ -carboxyl groups.) As far as the side-chain carboxyl groups are concerned, the non-zero value of w turns out to be simply the result of assuming, falsely, that the pK 's for aspartyl and glutamyl side chains are identical. When proper account is taken of the pK difference between these groups (see Table III) the acidic branch of the titration curve can be assigned a value of w which is zero within experimental error. The non-zero value of w for the phenolic groups, however, represents a real deviation from the ideally expected result. This probably does not mean that there continues to be, under these conditions, a general electrostatic interaction between all the charges on the molecule, as visualized in the Linderström-Lang treatment, but rather the existence of specific local effects which invalidate the idea that all groups of the same kind titrate altogether simultaneously. The titratable groups on a protein molecule, even when it is unfolded, have different environments by virtue of having different neighbors on adjacent residues. For instance, location of a charged group on a neighboring residue could affect the pK value even at high ionic strength. Or a neighboring disulfide bond could provide a somewhat hydrophobic environment, because separation of all parts of the chain from each other would not be complete at that point. In ribonuclease some such effect clearly influences the titration of the phenolic groups. Within the experimental uncertainty of the data of Fig. 4, no similar effect need be invoked for any of the other groups of the protein.

¹⁹ See footnote 1 (p. 99) for method of correcting for overlaps of the titration of groups of one kind with those of another.

III. Titration of Native Proteins

The simple result obtained for proteins in denaturing media will certainly not hold true for most native proteins, because they provide more complicated interactions due to their special, tightly folded structure of peptide chains. A considerable number of groups can always be expected to titrate anomalously in the native protein. The easiest way to demonstrate this is to measure the titration curve of a native protein at high ionic strength, where electrostatic effects must be greatly reduced. Nevertheless, large differences from the titration curves in 6 *M* guanidine hydrochloride can still be found, as is demonstrated dramatically by the data for β -lactoglobulin shown in Fig. 5. That these differences truly

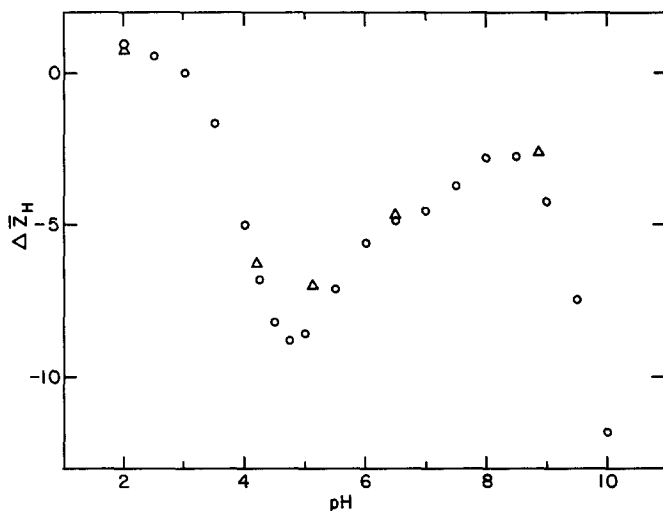


FIG. 5. Difference between the titration curves of β -lactoglobulin in 6 *M* GuCl and 1 *M* KCl at 25° (C. Tanford and Y. Nozaki, in preparation): ○ represents $\Delta\bar{Z}_H = \bar{Z}_H$ in 6 *M* GuCl - $\bar{Z}_H$ in 1 *M* KCl, at the same pH; Δ represents $\Delta\bar{Z}_H$ obtained by direct difference titration with a pH-stat.

reflect the difference between native and denatured conformations is demonstrated directly by difference titrations, as shown in Fig. 6. The difference is seen to arise during a steep transition over a narrow range of guanidine concentration, corresponding to the transition from native to denatured protein as observed by measurement of optical rotation or other conformation-dependent parameters.²⁰

Turning next to the actual analysis of the titration curves of native proteins, we begin with group counting. We ordinarily find differences

²⁰ N. Pace, unpublished data.

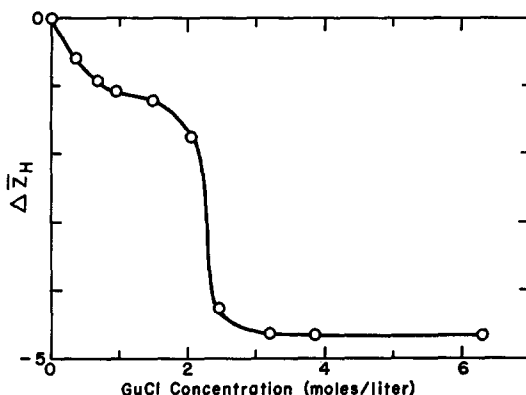


FIG. 6. $\Delta \bar{Z}_H$, measured as in Fig. 5 with a pH-stat, as a function of the concentration of GuCl at constant pH of 6.5. (C. Tanford and Y. Nozaki, in preparation). The solution at (GuCl) = 0 contained 1 M KCl, and the initial decrease in $\Delta \bar{Z}_H$ presumably reflects the increase in ionic strength above 1 M. The major part of the titration curve difference closely parallels the unfolding of the protein as observed by optical rotation or difference spectra.

between the number of groups titrated in a given region, and the number expected on the basis of amino acid analysis. For ribonuclease (Fig. 1), for example, we find that the expected number of groups is titrated in the acid and neutral branches, and also the measurement of ΣN^+ continues to give the expected result, as for the titration in 6 M GuCl. On the alkaline side, however, three fewer groups are titrated than expected from amino acid analysis. Spectrophotometric titration (Fig. 2) shows that the three groups not titrated are phenolic groups. Only 3 of the 6 such groups can be titrated at all in the native protein, while the other three are unavailable for titration until the native conformation is altered. In hemoglobin²¹ and in myoglobin,²² by contrast, carboxyl groups are normal, but some imidazoles are titrated in the acid branch of the curve instead of the neutral branch. We regard all unavailable groups of this sort as buried in the interior of the protein molecule, so that they are not titrated at all unless a conformational change in the protein molecule exposes them to the solvent. In many cases the conformational change required is drastic and irreversible. The buried groups in most cases are probably present as uncharged species. For instance, the three tyrosine groups of ribonuclease,²³ the buried histidine groups of hemoglobin and

²¹ J. Steinhardt and E. Zaiser, *J. Am. Chem. Soc.* **75**, 1599 (1955).

²² E. Breslow and F. R. N. Gurd, *J. Biol. Chem.* **237**, 371 (1962).

²³ C. Tanford, J. D. Hauenstein, and D. G. Rands, *J. Am. Chem. Soc.* **77**, 6409 (1955).

myoglobin, and the two abnormal carboxyl groups of β -lactoglobulin²⁴ are all present in the uncharged form. However, the existence of buried charged carboxylate groups has been reported for lysozyme.²⁵

There are also anomalies in pK which are not so drastic as the above examples, although they are related to the special conformation of protein molecules. For instance, in β -lactoglobulin two carboxyl groups titrate reversibly in the neutral region of the curve instead of the acidic region.²⁴ They appear to be buried in the native protein, and to become exposed at neutral pH in a conformational change which, although it can be detected by changes in optical rotation and other parameters, is much less drastic than complete unfolding. The behavior of these two groups, plus other anomalies not yet identified, lead to the large difference in titration behavior between native and denatured proteins shown in Fig. 5.

More subtle anomalies are usually observed when the groups titrated in the normal range are subjected to analysis by Eq. (9). From the logarithmic plots of Eq. (9) against $\bar{Z}$ for the carboxyl groups of ribonuclease, w values were found that were much higher than predicted by a spherical model for the protein. In this case the anomalous w values were found to stem from the fact that there must be at least two kinds of β,γ -carboxyl group with different pK^{int} values.^{26,27} The w value found for lysozyme is also much higher than predicted by a spherical model.²⁸ High w values are obtained quite often, and it is possible that most of them can be explained by the involvement of heterogeneous pK^{int} values among chemically identical groups.

Even when the analysis itself does not show obvious anomalies, there must be effects of conformation on the titration curve which are hidden by the averaging process that occurs when attempts are made to reconcile the experimental data on the assumption that all groups of a given kind titrate simultaneously. In hemoglobin, for example, there are at least two groups which must have different pK 's in the oxygenated and deoxygenated forms.^{29,30} The pH-dependence of the dissociation of hemoglobin into half-molecules requires that there must also be groups which have a different pK in the whole molecule and in the half-mole-

²⁴ C. Tanford, L. G. Bunville, and Y. Nozaki, *J. Am. Chem. Soc.* **81**, 4032 (1959).

²⁵ J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.* **82**, 2154 (1960).

²⁶ C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.* **78**, 5287 (1956).

²⁷ J. Hermans, Jr. and H. A. Scheraga, *J. Am. Chem. Soc.* **83**, 3293 (1961).

²⁸ C. Tanford and M. L. Wagner, *J. Am. Chem. Soc.* **76**, 3331 (1954).

²⁹ E. Antonini, J. Wyman, M. Brunori, C. Fronticello, E. Bucci, and A. Rossi-Fanelli, *J. Biol. Chem.* **240**, 1096 (1965).

³⁰ J. Wyman, Jr., *Advan. Protein Chem.* **19**, 223 (1964).

cule.³⁰ These anomalies cannot be detected with any assurance by examination of the titration curve per se, and no satisfactory method for identifying them exists.

Most of these anomalies have not yet been explained in terms of structure. All that can be said is that they are characteristic of the native conformation, and that they disappear when the native conformation is lost. In some instances, as in the case of oxy- and deoxy-hemoglobins, they point to a difference in conformation between two forms of the native protein.

IV. Experimental Technique

a. Glass Electrode. Requirements for a glass electrode are: (1) quick and stable pH response—a good electrode reaches the final pH reading quickly and shows no pH drifts for several hours thereafter; (2) $p\gamma'_H$ and $p\gamma'_{OH}$ values are constant over wide pH ranges, at least slightly beyond the pH limits within which titrations are intended, and ordinarily $p\gamma'_H$ should be constant to pH 1.5 and $p\gamma'_{OH}$ to pH 12.5, when determined in KCl solutions; and (3) durability, that is, the qualities described remain satisfactory for a long time. A good electrode will deteriorate eventually and rejuvenation methods usually do not restore its lost performance. Unfortunately not all commercial electrodes meet these requirements. Only one out of about three behaves satisfactorily.

b. Calomel Electrode. Calomel electrodes with finely drawn, U-bent capillary tips, as shown in Fig. 7B, are recommended for continuous titration. Commercial calomel electrodes that have a wick, porous plug, or sleeve to make the liquid junction are not satisfactory for protein solutions. The use of the U-bent tip was recommended by Jacobsen *et al.*³¹ and is commercially available. It is important, however, to make the capillary of the U-tip very fine,³² so that the amount of KCl flowing into the solution can be ignored.

c. Syringe Burette. The major problem with syringe-type microburettes is leakage. If the reagent is leaking into the sample solution (leaking forward), too flat a titration curve will be obtained; backward leakage will result in too steep a titration curve. In order to prevent leakage in the case of all-glass syringe burettes, the end piece and the barrel are fused together. In addition, a drop of glycerol is applied on the plunger at the rear end of the barrel after filling the syringe with a standard solution. A U-shaped wire is useful to prevent the plunger from

³¹ C. F. Jacobsen, J. Léonis, K. Linderstrøm-Lang, and M. Ottesen, *Methods Biochem. Anal.* **4**, 171 (1957).

³² Approximately 15/100 mm in diameter, or about the size of the bore of an AGLA syringe tip or Gilmont microburette glass tip.

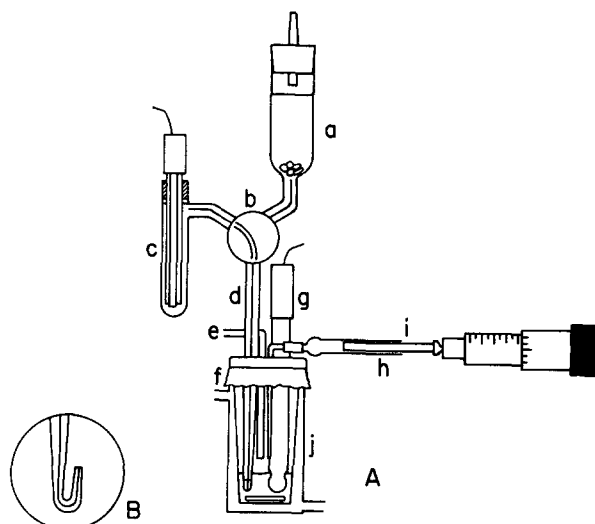


FIG. 7. Apparatus for continuous titration: (a, b, c, d) calomel electrode assembly (the salt bridge tip is shown in insert B). A commercial calomel electrode is cut near its tip and secured inside (c) with Tygon tubing (shaded). The three-way glass capillary stopcock is shown in working position. (e) Nitrogen gas inlet. (f) Rubber sheet (dental dam). (g) Glass electrode. (h) Syringe burette assembly. (i) Place of application of sealant glycerol. (j) Conical titration vessel with water jacket and a stirring bar. The whole assembly is placed on a magnetic stirrer.

slipping. Usually an L-shaped delivery tube is attached to the syringe through a short piece of Tygon tubing. The delivery tube is brought into the titration vessel only when there is no sign of leakage, forward or backward. Forward leakage can be detected by touching the syringe tip with the fingernail, on which a wet spot will be seen if the syringe is leaking. An air bubble will be noticed in the syringe tip, if backward leakage occurs.

In the case of other types of syringe burette, in which the plunger passes through plastic gaskets, possible leakage can be minimized by filling the syringe bubble-free.

d. Vessels for Continuous Titration. The titration vessels currently being used in the laboratory of the authors are made from solid plastic rod, so that any possibility of leakage is excluded. An interchangeable water jacket can be screwed on the inner vessel, and the sample can be weighed directly in the inner vessel before assembly. The smallest vessel for 2.5–3-ml samples is shown in Fig. 7A. Thorough mixing is essential to successful titration. It becomes difficult when a small-size, say 2.5-ml, sample is to be titrated, since a part of the solution trapped in the narrow

space might not be mixed well with the main body of the solution. Dixon and Wade have designed a novel titration vessel which is rotated by a constant temperature water jet.³³ Another small vessel has been designed by Leonis and Li for use with a 1-ml sample.¹⁵

e. Guanidine Hydrochloride. At present the quality of commercial guanidine hydrochloride is not satisfactory for titration work. It is recommended that the salt be recrystallized by the following procedure: 250 g GuCl is dissolved in 1 liter of hot absolute ethanol, and the solution is decolorized with charcoal and filtered through a heated large funnel if necessary. Benzene (500 ml) is added to the still hot ethanol solution, and the mixture is kept in the cold for several hours before the crystals (needles) are collected. Sometimes a yellow, sticky impurity can be removed from the original sample by washing with acetone.

The recrystallized sample should be further recrystallized either from methanol by dissolving in nearly boiling methanol and chilling in Dry Ice-acetone mixture, or from water by vacuum evaporation of an aqueous solution nearly saturated at 40°.

An alternative procedure is to neutralize a slurry of guanidinium carbonate, which has been recrystallized from 50% ethanol, with 20% distilled hydrochloric acid to pH 4. The solution is evaporated to obtain crystals of guanidine hydrochloride. The product is fairly pure, but still requires recrystallization as described above.

Criteria of purity are: (1) The absorption spectrum of a 6 *M* solution should show gradually increasing absorbance and no peak from 350 $m\mu$ to near 230 $m\mu$, where a sharp rise is usually observed to approximately 0.15 absorbance unit at 225 $m\mu$. (2) A minute amount of standard acid or base should cause a large pH shift. Quantitatively, titration of 6 *M* guanidine hydrochloride solution should produce $p\gamma'_H$ and $p\gamma'_{OH}$ values comparable with the values given in Table II.

f. Titration Procedure. Generally speaking, continuous titration is liable to systematic error, but the accuracy can approach that of batchwise titration if proper care is taken. It has the great advantage of saving time and material. The following comments apply mostly to continuous titration, although some are also relevant to batchwise titration technique.

It is recommended that a blank titration be performed occasionally, so that irregularities in electrodes or standard solutions can be detected before a protein sample is titrated. Attempts to determine $p\gamma'$ values with too low a concentration of acid or base added should be avoided. The concentration should be at least 3×10^{-3} moles per liter to maintain sufficient buffer capacity.

* G. H. Dixon and R. D. Wade, *Science* **127**, 338 (1958).

Choice of concentration of protein for titration will vary over a wide range, but the most convenient concentration is 0.5–1%, except at very low pH (<2.5) and high pH (>11), where the concentration of free H^+ and OH^- ions reaches the same order of magnitude as of bound ions and the accuracy of the titration will decrease unless a high protein concentration, say 3% or even higher, is used. This is especially desirable for accurate $\bar{Z}$ values near the acid end of titration curves, where the important value of ΣN^+ is obtained.

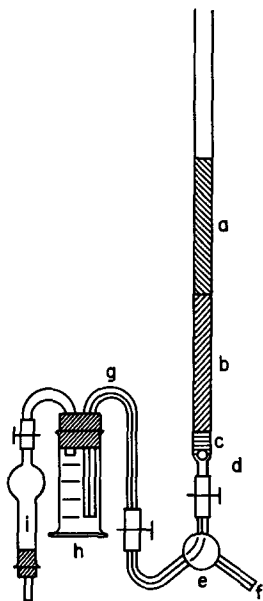


FIG. 8. Apparatus for deionization of protein solutions. (a) Mixture of ammonium and acetate forms of ion-exchange resins. (b) Mixture of hydrogen and hydroxyl forms of ion-exchange resins. (c) Short layer of hydrogen form of cation-exchange resin. (d) A glass bead. (e) Three-way Teflon capillary stopcock. (f) Capillary outlet. (g) Capillary U-tube. (h) Receiver. (i) Ascarite tube.

It is advisable to shift the pH of an initial sample solution in the direction opposite to that of the intended titration, so that at least a portion of different titration curves will be superimposed in a particular pH region. It is also recommended that nitrogen gas be passed over the solution at slightly acidic pH for a while until the pH reading becomes constant before a continuous titration starts, since it is possible that a sample of high pH will have absorbed CO_2 from the air.

g. Deionization and Isoionic Point Measurement. Dintzis³⁴ has introduced a convenient procedure to deionize proteins which has become

³⁴ H. Dintzis, Ph.D. thesis, Harvard University, Cambridge, Massachusetts, 1952.

standard. A diagram of the apparatus believed to be most convenient is shown in Fig. 8.

After packing the column with three layers of resins, as shown in the figure, it is rinsed repeatedly with water. The conductivity of the rinse water can be determined by attaching a flow cell to *f*, while the receiver *h* may be flushed with nitrogen gas. The sample solution is placed on the column when the rinse water is about to disappear. The effluent does not contain an appreciable amount of protein until the volume reaches roughly half the apparent volume of the resin column. Usually an amount of effluent is collected equal to about 1.5 times the initial volume, depending on the retention of protein by the resin, to recover nearly all the protein. Roughly speaking, 9 ml resin (2 ml each of hydrogen, hydroxyl, ammonium, and acetate cycles, plus 1 ml hydrogen resin at the bottom) is sufficient to retain 10^{-3} equivalent of salt. The flow rate is kept rather slow, about 1 ml per minute for a column containing 9 ml resin.

It is recommended that untreated samples as well as samples adjusted to acid and alkaline pH, if the quantity of protein permits, be deionized to confirm the validity of the pH values obtained. If the column is working properly, the final pH will be independent of the initial pH.

[85] Hydrogen Exchange

By GIOVANNI DI SABATO and MARTIN OTTESEN

I. Introduction

It is known that when proteins are dissolved in deuterium oxide some of the hydrogen atoms will exchange for deuterium. Linderstrøm-Lang developed a quantitative method to determine this exchange, and demonstrated that in simple peptides and in denatured proteins the hydrogens bound to nitrogen, to oxygen, or to sulfur would exchange rapidly.¹ In native proteins, structural factors were present that slowed down the exchange. In most cases some of the exchange was so slow that some potentially exchangeable hydrogens remained unexchanged after several days in deuterium oxide solution at ordinary temperatures. Therefore, measurements of the rates and extent of hydrogen exchange in native proteins provide information about their structure. In the present article the mechanism of the hydrogen exchange is not discussed in detail, but

¹I. M. Krause and K. Linderstrøm-Lang, *Compt. Rend. Trav. Lab. Carlsberg*, **29**, 367 (1955).