Hydrogen Ion Titration Curve of Lysozyme in 6 M Guanidine Hydrochloride*

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ABSTRACT: The hydrogen ion titration curve of lysozyme in guanidine hydrochloride is shown to be completely predictable on the basis of model compound pK values in that solvent. The acidic amino acid content of our sample was ten per mole of protein, one less than the number found in sequence studies.

It has been established that most proteins, with disulfide bonds reduced, exist as linear random coils in concentrated Gdn·HCl[1] solutions. When disulfide bonds are intact, many proteins, including lysozyme, appear to be cross-linked random coils in this solvent, *i.e.*, they are devoid of noncovalent interactions leading to ordered structures, even though the disulfide bonds limit the extent of expansion of the molecule. The effective size, as determined from hydrodynamic measurements, is therefore considerably smaller than for a randomly coiled linear polypeptide chain of the same number of amino acid residues.

One test for the absence of ordered structures is to measure the hydrogen ion titration curve of the protein. Short-range interactions leading to anomalous titration behavior should be absent. Where the denaturant is a strong electrolyte, as Gdn·HCl is, long-range electrostatic interactions should also become insignificant. The titration curve thus becomes completely predictable, each titratable group behaving independently of all others, with a pK identical with that determined from a suitable model compound of low molecular weight.

The experimental titration curve of ribonuclease in 6 M Gdn·HCl is in agreement with such a prediction, except that small deviations from the expected behavior, barely outside experimental error, were noted for the phenolic hydroxyl groups of tyrosine side chains (Nozaki and Tanford, 1967b). These deviations were not considered to indicate the presence of ordered structures, but were thought to be due to nonideality of the randomly coiled polypeptide chain: *i.e.*, conformations of the chain in which highly hydrophobic portions, such as the tyrosyl side chain, are in mutual contact would be slightly favored over conformations in which they are fully exposed to solvent. As a result, the average environment of some phenolic groups might deviate to a measurable extent from that of a model compound in the same solvent. Another small anomaly, time dependent and irreversible, was observed in ribonuclease at pH values above pH 10.5, and it was ascribed to the slow attack of OH^- ions on disulfide bridges.

The present paper reports a similar experimental study for lysozyme. The experimental titration curve will be seen to be in almost exact agreement with the theoretically predicted one. No anomalous behavior was found in the titration of the phenolic groups. The time-dependent anomaly ascribable to the attack of OH^- ions on disulfide bridges was again observed.

Materials and Methods

Protein and Protein Solutions. Hen's egg white lysozyme, recrystallized three times as the chloride, was purchased from Pentex Inc., lot numbers 19 and 28, and purified further by ion-exchange chromatography on IRC-50 according to the procedure of Tallan and Stein (1953). The fractions to be used were then concentrated in a positive pressure membrane concentrator using Amicon UM-10 membranes and dialyzed, in the cold, against several changes of distilled water, over a period of 24 hr, in acetylated dialysis bags. The retained protein solution, at a concentration below 10 mg/ml, was run through a mixed-bed deionizing column, as described by Nozaki and Tanford (1967c), which was monitored by conductivity using a Radiometer CDM2d conductivity meter with a flow cell. The effluent, which emerges at a pH 10–11, depending on the protein concentration, was adjusted to pH 5 with 0.1 M HCl and lyophilized. This product was used in all experiments.

Protein stock solutions were made up at high protein concentrations in water and cleared by centrifugation if turbid. Concentration was determined by measuring optical density at 281 nm of an appropriate dilution of the stock solution made by weight with 0.1 M KCl. Dilutions were by a factor sufficiently large that the final salt concentration was not significantly less than 0.1 M. Optical density measurements were made in a Cary 15 spectrophotometer and were not corrected for light scattering.

The appropriate extinction coefficient was determined by evaporating to dryness a stock solution whose optical density had been measured as described. The residue from the water used in making the stock solution was insignificant, being on the order of 1 mg/l., so the residue on drying was assumed to be entirely due to protein plus chloride counterions. Evaporation was carried out at 107°, in air, in a forced draft oven. The weight of a 2.5-g sample reached a minimum within 7 hr and remained constant within 1 mg for 24 hr.

Solutions of protein, to be titrated, were made by weight, adding protein stock solution to stock solutions of KCl or Gdn·HCl allowing for the dilution of both proteins and solvent.

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[1] Abbreviation used is: Gdn·HCl, guanidine hydrochloride. This abbreviation is being used contrary to the authors' wishes.

Standard Acid and Base Solutions. Constant-boiling HCl, prepared according to the procedure of Foulk and Hollingsworth (1923), was used to make standard HCl solutions.

Carbonate-free KOH solutions were made according to the procedure of Powell and Miller (1957) and standardized by potentiometric titration against standard HCl solutions. KOH solutions were stored in Pyrex bottles equipped with a siphon and protected by trap containing "Ascarite"; a solid CO_2 adsorbent, obtained from Arthur Thomas Company, Philadelphia, Pa.

A single pH standard, potassium dihydrogen phthalate, NBS sample 185d, having a pH of 4.008 at 25° in 0.05 M solution, was used throughout.

Guanidine Hydrochloride. Guanidine hydrochloride purchased from Heico, Inc., was found to be free of titratable impurities, to have a low absorbance down to 225 nm, and to have a sharp melting point at 287–287.5° (Wong *et al.*, 1971) and was used without further purification. Concentration of stock solutions was determined refractimetrically (Kielly and Harrington, 1960).

Titration Measurements. Continuous titrations were performed using the technique and apparatus described by Nozaki and Tanford (1967a) with Corning glass electrode (catalog no. 476022), a homemade calomel electrode, and a Radiometer pHm4c pH meter. In experiments with alkaline solutions the vessel was flushed with nitrogen that had been passed through ascarite and bubbled through a solution whose vapor pressure was close to that of the solution being titrated. This is extremely important in Gdn·HCl titrations because a 6 M Gdn·HCl solution is only about 50% water and therefore has a very low vapor pressure. If the titration vessel is flushed with nitrogen equilibrated at the vapor pressure of pure water, sufficient condensation into the titration solution will occur over the period of a few hours to significantly alter the Gdn·HCl concentration and hence, the apparent activity coefficient. This will lead to spurious results at the ends of the titration curve where the activity coefficient correction becomes important. For measurement of the spectrophotometric titration curve solutions of varying pH were made by adding weighed amounts of stock solutions of protein in water or Tris buffer, Gdn·HCl, 1 M KOH, and 1 M KCl, the last so as to bring the total volume to about 4.0 ml. The difference spectrum of each solution was measured against a matching solution at pH 6. Since substitution of KOH for KCl resulted in no difference spectrum in the absence of proteins in the wavelengths interval between 340 and 290 nm, tandem cells were not used. After measuring the difference spectrum, the pH of the solution was measured. No attempt was made to exclude CO_2 from these solutions since the pH measurement is not used in the calculation of binding.

A time-dependent change in absorbance was observed at higher pH which was corrected for by extrapolating to zero time. No corresponding time dependence of the pH measurement was observed.

All measurements were made at total optical densities of about 0.5, the maximum ΔOD then being about 0.1. Spectral measurements were made using a Cary 15 spectrophotometer.

All pH measurements were made at 25.00°. The spectral measurements were made at room temperature which was 24 ± 2°.

Preparation of Isoionic Solutions. Isoionic protein solutions were prepared using the same deionizing column which was used in the purification procedure. The effluent was collected in a nitrogen-filled flask protected with an ascarite tube. Protein concentration was measured as on other protein stock solutions except that the diluent was 0.1 M KCl–0.005 M KH_2PO_4, the buffer being included to bring the pH below the range of tyrosyl ionization. The deionized protein stock solution was then added to saturated Gdn·HCl solution. The pH of these solutions was measured in the batchwise apparatus while flushing with nitrogen.

Amino Acid Analyses. Amino acid analyses were performed after 24- and 48-hr hydrolysis times on a Beckman 120-B amino acid analyzer.

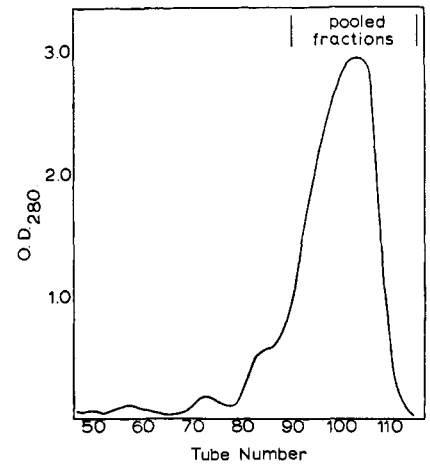

FIGURE 1: Elution pattern of Pentex lysozyme on IRC-50, after the procedure of Tallan and Stein (1953).

Results

Protein Purification and Extinction Coefficient. The 3x-recrystallized lysozyme as purchased was found to separate on IRC-50 into four distinct fractions as shown in Figure 1, the largest and slowest moving of which accounts for 85–90% of the area under the chromatogram and appears to be somewhat skewed toward the slow side of the peak. This elution pattern is in general accord with those observed by Tallan and Stein (1953) who showed that all of the peaks contain lysozyme activity and suggested that the faster running peaks are deamido products of the major component.

Deionized, lyophilized protein from the pooled fractions of the preparative run was rechromatographed on a scaled-down IRC-50 column. As shown in Figure 2, a single peak is

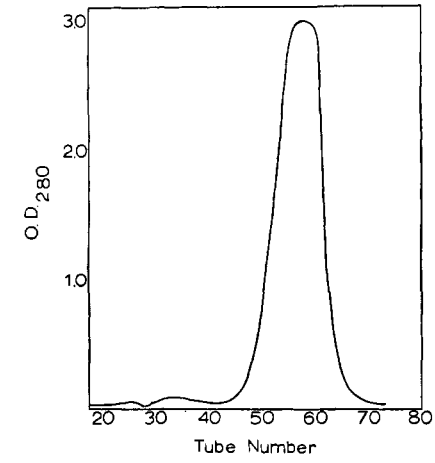

FIGURE 2: Lysozyme, purified for titration studies, rechromatographed on IRC-50.

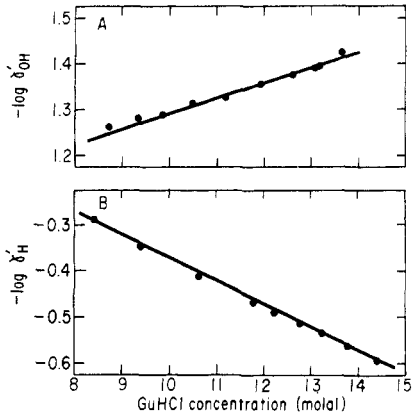

FIGURE 3: Apparent activity coefficients of hydroxyl ion (A) and hydrogen ion (B) in Gdn·HCl.

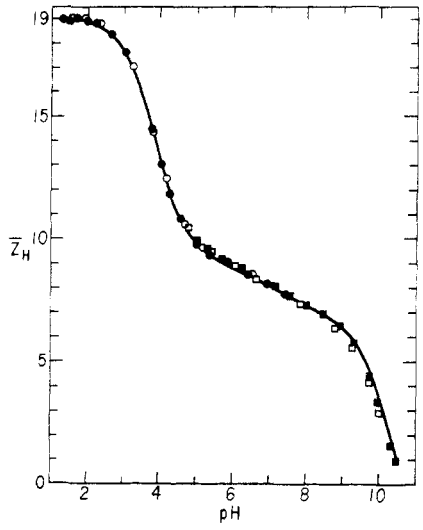

FIGURE 4: Hydrogen ion titration curve of lysozyme in Gdn·HCl. Measurements in acid region: (●), titration from neutrality to acid end; (○), reverse titration from acid end to neutrality. In basic region: (■), titration from neutrality with base; (□), reverse with acid. Results from the two experiments were superimposed empirically in the overlap region. The line is the theoretical curve calculated by eq 1.

eluted which appears more symmetrical than that from the preparative column.

The deionization step in the purification procedure was considered to be necessary to ensure that all buffer salts used in the chromatography were removed from the protein. Tallan and Stein found that, in contrast to similar procedures on ribonuclease, deionization of lysozyme with mixed bed resins extensively altered the chromatographic patterns, broadening the major peak and producing faster running material. The chromatogram in Figure 2 gives no indication that this is happening in our procedure.

To allay suspicions that the assymetry observed in the major peak in the preparative chromatography is due to the presence of an unresolved component, this peak was divided into two fractions which were carried through the rest of the purification procedure separately. These fractions were found to have identical titration curves in Gdn·HCl. The extinction coefficient for lysozyme at a concentration of 1 mg/g of solvent (0.1 M KCl) in a 1-cm cell was determined to be 2.74. The stock solutions on which these measurements were made had a pH of 5–6, at which lysozyme has a charge of +10 and will therefore be associated with 10 chloride counterions per molecule when prepared as described. Correction for this was made when the concentration was determined by dry weight.

Apparent Activity Coefficients. Apparent activity coefficients of H^+ and OH^- ions were determined as a function of Gdn·HCl concentration using the continuous technique. The solvent was first titrated to low or high pH with acid or base, and then diluted progressively with water. The concentration of the ion in question was kept higher than 10^{-3} M because at lower concentration difficulty is encountered in measuring pH due to low buffering capacity. Linear plots of $-\log \gamma_H'$ and $-\log \gamma_{OH}'$ are obtained over the range of Gdn·HCl concentration from 9 to 14 *m* as seen in Figure 3. The values obtained agree well with those of Nozaki and Tanford (1967a) which indicates that the Gdn·HCl sample is free of titratable impurities.

Protein Titration. The potentiometric titration results are presented in Figure 4. These data are the result of two continuous titration experiments, one from pH 7.4 to the acid end point and reverse, and the other from pH 5 to alkaline pH and reverse. The two experiments are reconciled by superimposing the data in the overlap region.

Continuous titration experiments measure the difference in proton binding at a given pH from that in the starting solution; to establish absolute numbers of protons bound a reference point is required (Tanford, 1962). In this case it was assumed that the maximum positive charge due to bound protons ($\bar{Z}_{H,max}$) is equal to 19, corresponding to the number of basic groups per protein molecule.

The titration curve in Gdn·HCl is completely reversible in the acid region but appears to be irreversible in the alkaline region. When a solution is titrated from around neutral pH to pH 11 and back to neutrality, the reverse curve lies below the forward curve, rejoining the upward curve at about pH 7. Nozaki and Tanford (1967b) also observed this in the titration curve of ribonuclease in Gdn·HCl. This phenomenon is likely to be connected with the alkaline hydrolysis of disulfide bonds which has been shown to occur in proteins and model compounds (Bohak, 1964; Donovan and White, 1971). While the mechanism of this reaction is in some doubt, it is generally agreed that thiol groups, which would then titrate with p*K* values around 9, and lysinoalanine are produced. Nozaki and Tanford (1967b) observed a peak running in the amino acid analyzer in the same position as lysinoalanine in ribonuclease samples which had been titrated to pH 11 in Gdn·HCl. That the reverse titration curve from high pH lies below the forward curve and rejoins it, favors mechanisms which include the dissociation of a proton. Generally speaking, the conditions under which alkaline hydrolysis of disulfides has been observed in proteins are more severe than those encountered here, but the unfolding of the protein would be expected to enhance their lability by increasing their exposure to solvent.

The difference between upward and reverse titration curves increases with the maximum pH reached and the time spent at high pH. In one experiment a solution of lysozyme in Gdn·HCl was twice titrated to pH 10.5 and reversed. The second upward titration closely followed the previous reverse titration until pH 10 was reached. This suggests that the reaction responsible for the irreversibility does not begin until this pH is reached and that the titration curves are reversible to this point. In the experiments from which the data of Figure 4 were taken, care was taken to minimize the time spent at high pH and the difference between forward and reverse curves held to about 0.3 group.

TABLE I: Apparent pK's for Calculating Titration Curve of Lysozyme in Gdn·HCl.

Class of Group	pK'_{int}
α-Carboxyl	3.4
β-Carboxyl	3.9
γ-Carboxyl	4.35
Imidazole	6 5
α-Amino	7.6
Phenolic	9.9
ϵ-Amino	10.35
Guanidinium	12.5

Isoionic Point Measurement. The average net proton charge per protein molecule at the isoionic point, $\bar{Z}_{H,iso}$, can be determined in solutions made by adding neutral salt to deionized protein solutions according to the equation (Tanford, 1962)

$$C_p\bar{Z}_{H,iso} + C_{H^+} - C_{OH^-} = 0 \quad (1)$$

where C_p is the protein concentration and C_{H^+} and C_{OH^-} are the free concentrations of the respective ions. If an isoionic solution is titrated to the acid end point the maximum net charge, $\bar{Z}_{H,max}$, equal to the number of basic amino acids in the protein molecule, can be determined by the relation $\bar{Z}_{H,max} = \bar{Z}_{H,iso} + \Delta\bar{Z}_H$ ($\Delta\bar{Z}_H = \Delta\bar{\nu}_H$), where $\Delta\bar{\nu}_H$ is the number of additional protons bound in the titration. In measuring the isoionic reference point of lysozyme in Gdn·HCl, the difficulty is encountered that it lies at high pH, around pH 10, in the region where time-dependent changes occur. Consequently, the accuracy of the determination of $\bar{Z}_{H,iso}$ is poor.

Two experiments to determine $\bar{Z}_{H,max}$ were carried out. In the first, Gdn·HCl was added directly to the deionized protein solution and titration to the acid end point carried out. This gave a value of 18.6 for $\bar{Z}_{H,max}$. In the other experiment the isoionic solution was titrated in water to pH 4 before adding Gdn·HCl. This solution was then titrated to the acid end point giving a value of 18.7 for $\bar{Z}_{H,max}$. Thus there is some small disagreement with the choice of $\bar{Z}_{H,max} = 19.0$ as a reference point which is probably due to error in measuring the pH of the isoionic solutions. The form of these titration curves in the acid region was identical with that in Figure 4.

Theoretical Calculations. If the titration curve in Gdn·HCl is in fact unperturbed by any interactions, it can be calculated as the sum of the titration curves of individual groups according to the equation $\bar{Z}_H = \sum_i n_i\xi_i$, where n_i is the number of groups of type i and ξ_i is the fractional charge. The fractional degree of saturation, X_i, is, at given pH, $X_i = 10^{pK_{int,i}-pH}/(1 + 10^{pK_{int,i}-pH})$, where $pK_{int,i}$ is the intrinsic pK in Gdn·HCl, and $\xi_i = X_i$ for basic groups, $\xi_i = 1 - X_i$ for acidic groups. The line drawn through the data in Figure 4 was obtained with these equations, the pK_{int}' given in Table I, and the n_i given in the last column of Table II.

It will be seen that the number of carboxyl groups assumed in this calculation is one fewer than the number found to be present by Canfield (1963). The fit obtained with this assumption is very good and addition of another carboxyl group to the calculated cruve would place it well outside of the experimental error of the measured curve. It has been assumed that the "missing" carboxyl group is an Asp rather than a Glu solely on the basis of mathematical probability, making the calculation with 8 Asp and 1 Glu would fit the data as well.

TABLE II: Amino Acid Compositions of Lysozyme.

Residue	Number Found by Analysis of Purified Pentex Sample	Number Expected (Canfield, 1963)	Number Assumed in Calculations
Asp	{20.7	8	7
Asn		13	
Glu	{ 5.0	2	2
Gln		3	
His	0.9	1	1
Tyr	2.8	3	3
Lys	6.0	6	6
Arg	11.1	11	11
α-COOH		1	1
α-NH_2		1	1

The results of the spectrophotometric titration of lysozyme are shown in Figures 5 and 6. The line in Figure 5 is drawn assuming a total change in extinction at 297 nm, $\Delta\epsilon_{297}$, equal to 7350 and a pK of 9.9. The value of $\Delta\epsilon_{297}$/mole of tyrosine would then be 2450. The same data plotted as $\log[\alpha/(1-\alpha)]$, where α is the degree of dissociation, are shown in Figure 6. The linearity of this plot from $\alpha = 0.02$ to $\alpha = 0.98$ attests to the constancy of the pK over this range.

The open circles in Figure 5 are points determined in the absence of buffer. At pH above 10 they agree well with the measurements made in the presence of 0.01 or 0.005 M Tris buffer, but deviate significantly from these measurements at lower pH. This effect is due to the low buffering capacity of the protein solutions alone which contained only 0.6 mg/ml or 10^{-5} M lysozyme. At concentrations of free H^+ or OH^- below about 10^{-3} it becomes difficult to measure the pH accurately in the absence of buffer.

Discussion

The titration curve of lysozyme in Gdn·HCl conforms completely to the pK_{int}' expected on the basis of model compound pK's in this solvent (Nozaki and Tanford, 1967a). There is, however, a discrepancy between the number of carboxyl groups titrated, 10, and the number expected on the basis of sequence studies, 11 (Canfield, 1963). This difference is well outside the experimental error of our measurement, and since it is unlikely that residual interactions could shorten the titration curve by exactly one group without detectably perturbing other pK's, we conclude that ten carboxyl groups is the correct number for our sample. Since the amino acid composition includes the expected number of glutamic acid + glutamine and aspartic acid + asparagine residues, it appears that an amide is substituted for an acidic residue. Since there are only two glutamic residues, and one of these is postulated to participate as such in the enzymatic mechanism (Rupley *et al.*, 1967; Blake *et al.*, 1967), the substitution is assumed to be an asparagine for an aspartic acid.

This is a puzzling but not a unique observation, for a differ-

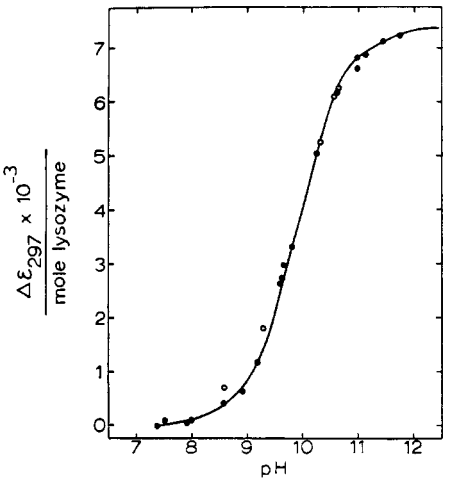

FIGURE 5: Spectrophotometric titration curve of lysozyme in Gdn·HCl. Open circles represent measurements made in the absence of buffer, filled circles in the presence of Tris buffer. The line represents the theoretical curve for three identical groups, each titrating with a pK of 9.9 and having $\Delta\epsilon$ = 2450 per mole of tyrosine.

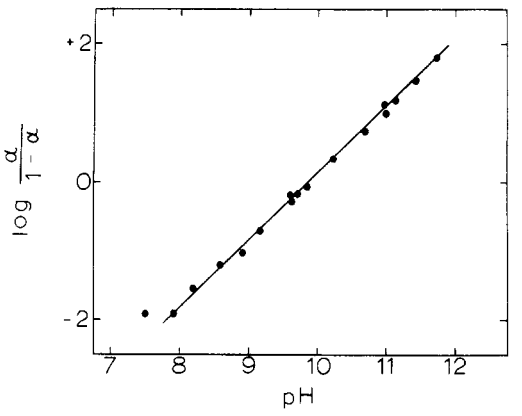

FIGURE 6: The data of Figure 8 plotted as log $X/(1 - X)$, where X is the fractional saturation.

ent carboxyl group number has been found in each sample of lysozyme that has been titrated. The sample titrated by Donovan *et al.* (1960) had 12, while that titrated by Tanford and Wagner (1954) apparently had 14, although, not being aware of the presence of the very low pK groups in this protein, the latter authors reported 12. Measurements made by Donovan *et al.* (1960) on the sample used by Tanford and Wagner revealed the extra two groups. Sakakibara and Hamaguchi (1968) found 11 carboxyl groups in their sample. The observed variability may be related to the finding by Tallan and Stein (1953) that the faster running fractions in the chromatographic procedure, which have enzymatic activity and are suggested to be desamido products of lysozyme, vary in amount depending on the history of the sample and, in particular, on the type of anion present.

With one exception, the pK_{int}' used to calculate the theoretical titration curve in Figure 4 are within ±0.1 of those used by Nozaki and Tanford (1967b) to calculate the titration curve of ribonuclease in Gdn·HCl. The exception involves the tyrosyl residues, which, in ribonuclease, did not titrate with a single pK. To fit the spectrophotometric data it was necessary to assume that three of the groups had slightly elevated pK's of 10.15. No such anomaly was observed in the lysozyme data, the single observed pK of 9.9 agreeing well with the expected value of 9.8 ± 0.1.

Acknowledgment

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