Thermodynamics of the Denaturation of Lysozyme by Guanidine Hydrochloride. I. Dependence on pH at 25°*

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ABSTRACT: The denaturation of hen egg-white lysozyme has been studied at 25°, as a function of the concentration of guanidine hydrochloride and of the pH. The results of this study show that the pH dependence between pH 1 and 4 is primarily due to two acidic groups that have abnormally low pK's in the native state. The known three-dimensional structure of the protein, together with literature data on the effects of pH on saccharide binding, suggest that the two groups may be glutamic acid residue 7 and aspartic acid residue 52 or 103. To account for the pH dependence between the pH range covered by this study and earlier data at pH 5.5 requires the presence of additional abnormal acidic groups in the native protein, one of these being the carboxyl group at residue 35, which has been suggested as having an abnormally high pK on the basis of studies of saccharide binding and enzyme kinetics of lysozyme.

A previous paper from this laboratory (Tanford *et al.*, 1966) has demonstrated that the denaturation of lysozyme by guanidine hydrochloride at pH 5.5 and 20–30°, is a reversible two-state process. This paper is concerned with the pH dependence of this process. It was found that the transition is reversible and a two-state process to very low pH, and that it shifts to lower guanidine hydrochloride concentrations as the pH is reduced. A detailed analysis of this shift has been made, and a quantitative interpretation is presented.

Experimental Procedure

Materials. The lysozyme used in this study was three-times-recrystallized hen egg-white lysozyme, lot no. 19, obtained from Pentex, Inc. The protein was quite pure as received, but was submitted to further purification according to the method of Tallan and Stein (1953).

Stock solutions were prepared from the purified protein so as to contain 0.1 M KCl. The final pH was generally near pH 4. Protein concentrations were determined by dry weight analysis. It was found that the protein has an absorptivity, $E^{1\%}_{1\,cm}$, at 281.5 mμ of 26.4, and this was used to determine protein concentrations for some of the stock solutions. Stock solutions were usually not employed for more than 2 weeks, but physical parameters such as concentration, absorption spectrum, and optical rotation were usually unchanged after as long as 1 month in the refrigerator.

The GuHCl[1] used in this study was obtained from J. T. Baker Co. and purified according to Nozaki and Tanford (1967).

Methods. Absorption spectra were measured using a Cary 15 double-beam recording spectrophotometer equipped with a sample and reference temperature control. The reference and sample were always maintained at 25.0°.

Optical rotation data were obtained using a Cary 60 recording spectropolarimeter, also equipped with sample temperature control maintained at 25°. All optical rotation data were reduced to the concentration-independent parameters $[\alpha]_\lambda$, the specific rotation, and $[m']_\lambda$, the mean residue rotation, the subscript λ denoting the wavelength at which the parameter is evaluated. The values of $[m']_\lambda$ were obtained from the relation

$$[m']_\lambda = \frac{3}{n^2_\lambda + 2}\frac{M_0}{100}[\alpha]_\lambda \qquad (1)$$

where n_λ is the refractive index of the solvent at λ mμ and M_0 is the mean residue weight (110.9 for lysozyme).

The values of n_{589} for water and 0.1 M KCl are essentially identical. We have assumed this identity at all wavelengths. The values of n_λ for GuHCl solutions are thus far available only at 589 mμ. To obtain values for n_λ at other wavelengths we have used the procedure described by Hooker (1966).

Measurements of pH were made utilizing a Radiometer 22 pH meter in conjunction with a Radiometer scale expander. Radiometer glass electrodes type G202B were used with a Radiometer K100 saturated calomel electrode as reference. The electrodes were standardized using Beckman standard buffers. For the acid region, with which most of this work is concerned, pH 4.005 potassium acid–phthalate buffer (at 25°) was the standard. No temperature control was employed for pH measurements.

Results

All available information indicates that native lysozyme is an unusually stable protein, and undergoes no significant conformational transformations, at 25°, in the absence of denaturant, within the pH range covered by the studies reported here. The starting material in all the transitions reported here is therefore the native protein, N, with a structure that is presumably very close to (perhaps identical with) the

* From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina. *Received July 16, 1969.* This paper is abstracted from the Ph.D. dissertation of K. C. A., Duke University, 1968. The work was supported by research grants from the National Institutes of Health, U. S. Public Health Service, and from the National Science Foundation.

[1] Abbreviation used is: GuHCl, guanidine hydrochloride.

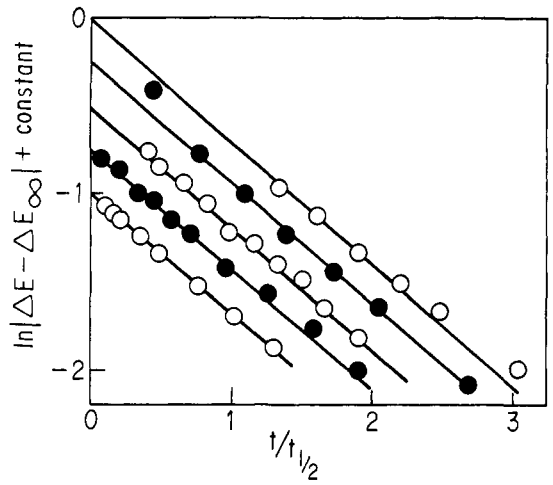

FIGURE 1: Kinetic data for denaturation and renaturation of lysozyme at several pH values and GuHCl concentrations, as measured by difference spectroscopy at 301 mμ. Open circles represent denaturation, filled circles renaturation. The lines, from top to bottom, represent pH 1.31, 3.20 M GuHCl, $t_{1/2}$ = 17.5 sec; pH 2.65, 1.92 M GuHCl, $t_{1/2}$ = 31.5 sec; pH 2.64, 2.50 M GuHCl, $t_{1/2}$ = 120 sec; pH 2.44, 2.58 M GuHCl, $t_{1/2}$ = 159 sec; pH 4.66, 4.01 M GuHCl, $t_{1/2}$ = 1480 sec.

structure of the native protein in the crystalline state (Blake *et al.*, 1965).

The end product of the denaturation (which will be designated as state D), obtained at high concentrations of GuHCl, is a randomly coiled polypeptide chain, cross-linked by four disulfide bonds, but retaining no fixed noncovalent interactions. As has been pointed out elsewhere (Tanford, 1968), the effective volume of lysozyme under these conditions is relatively small, and this means that the residues are closer to each other than in most randomly coiled proteins, and that the number of transient contacts between side chains and/or peptide groups is probably relatively large. The influence of such contacts on properties of individual residues, such as p*K*'s of acidic groups, is likely to be small. It will in fact be assumed that the titratable groups are fully exposed to the solvent. In addition, because of the fact that all equilibrium data used in this work were obtained at a GuHCl concentration of at least 1 M, the effects of electrostatic interactions between charged groups can be ignored as being insignificant (Nozaki and Tanford, 1967). The over-all conclusion is thus that the conformation D is the same at all pH values: only the state of titration of acidic and basic groups will vary.

Absence of Stable Intermediate States. The process we are studying is the equilibrium between unique native and denatured states

$$N \rightleftharpoons D$$

In order to extract usable data for the equilibrium in this process from the experimental data to be presented, it is necessary that no states other than N and D contribute to the measured properties during the gradual shift of the equilibrium from 100% N to 100% D.

A decisive test for the presence of stable intermediates is to study the kinetics of denaturation or renaturation, especially in the transition region, where the final product is an equilibrium mixture of N and D, and of other states, X_1, X_2, etc., if such contribute to the final equilibrium. If N and D are the only stable states, the kinetics will generally be first order in both directions, *i.e.*, only the processes N → D and D → N will be observed. Where y is the value of an observable parameter at any time, t, and y_0 and y_∞ are the values at zero time and at equilibrium, respectively

$$(y - y_\infty) = (y_0 - y_\infty)e^{-(k_1 + k_2)t} \qquad (2)$$

k_1 and k_2 being the rate constants for the process N → D and D → N, respectively. If, on the other hand, states other than N and D exist in the equilibrium mixture, then the rate will depend on the rate constants for the processes N → X_1, X_1 → D, D → X_1, X_1 → N, etc., and, under these circumstances, the kinetics are governed by a more complex relation

$$(y - y_\infty) = C_1e^{-\lambda_1 t} + C_2e^{-\lambda_2 t} + \ldots \qquad (3)$$

where the λ_i are functions of the various rate constants and the C_i are functions of these rate constants and of the physical properties of the states X_1, X_2, etc. Equation 3 could fortuitously reduce to eq 2 at a particular pH and a particular concentration of GuHCl, if for example, there are only two terms and if $\lambda_1 = \lambda_2$. Since at least some of the rate constants must depend strongly on pH and GuHCl concentration, such an equality could not hold over a wide range of conditions. As has been shown elsewhere (Tanford, 1968), a general reduction of eq 3 to eq 2 can occur only if all states other than N and D are in fact transient intermediates, making no contribution to the equilibrium properties of the system.

The test for the absence of stable intermediate states consists then of a plot of ln $|y - y_\infty|$ *vs.* time. If no stable intermediates exist, all such plots will be linear, and, in addition, the value of ln $|y - y_\infty|$, extrapolated to zero time, should be equal to the value of ln $|y_N - y_\infty|$ or ln $|y_D - y_\infty|$, depending on whether the initial state ($t = 0$) is the native or denatured protein.[2]

Measurements of this kind have already been reported for the GuHCl denaturation of lysozyme at pH 5.5 (Tanford *et al.*, 1966). No evidence for stable intermediates was found. Results obtained in this study at lower pH values agree with this conclusion. Figure 1 shows typical kinetic plots, using difference spectral measurements, and they are seen to be linear. Furthermore, the intercepts at $t = 0$ all agree, within experimental error, with the expected values of y_N or y_D for each run. The intercepts at $t = 0$ for three kinetic runs in each direction are shown, as examples, by the circumscribed *K*'s in Figure 3.

The absence of any contribution to the measured properties from states other than N and D means that all of the data in Figures 2 and 3 represent solely the equilibrium N ⇌ D,

[2] It is possible that the effective rate constants λ_i of all but one of the terms of eq 3 are so large that the time dependency contributed by all such terms is complete before the first measurement is made. In that case plots of ln $|y - y_\infty|$ versus time would be linear, but they would not extrapolate to the proper intercept at $t = 0$.

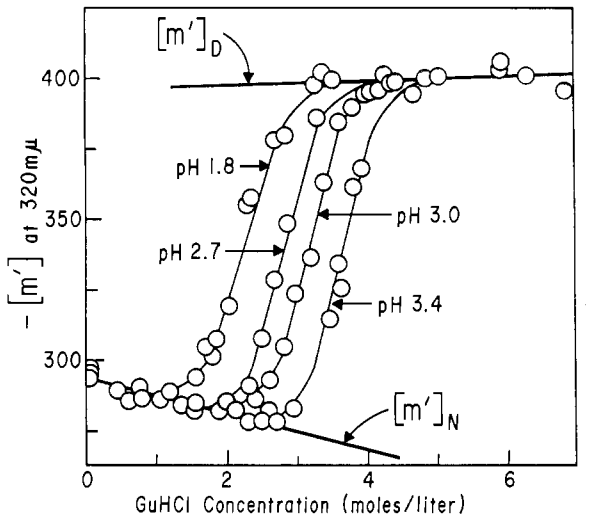

FIGURE 2: The effect of GuHCl concentration on the optical rotation at 320 mμ, at different pH values. The pH values assigned to each curve correspond roughly to the middle of each transition region. Measured pH values were used for all calculations. The actual pH ranges for points in the transition zone were 1.72–1.85, 2.66–2.76, 2.94–3.06, and 3.35–3.48 for the four experiments. The base lines, giving [m'] values for states N and D, are average values for the entire range of pH. The effect of pH on these rotations was measured, but is too small to be significant.

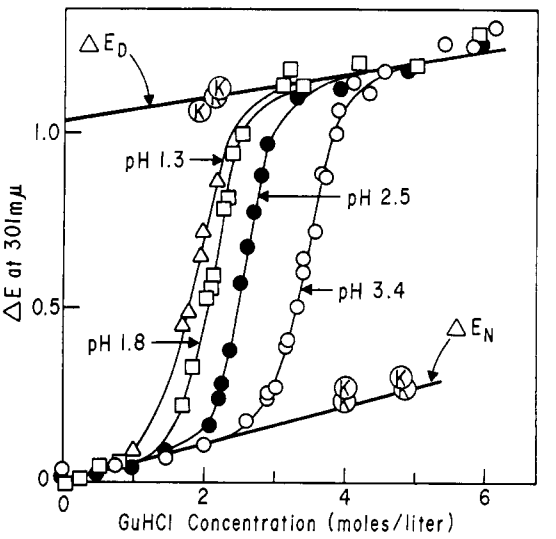

FIGURE 3: The effect of GuHCl concentration on difference spectral intensity at 301 mμ. The pH values have the same meaning as in Figure 2. Actual pH ranges in the transition zone for the four curves were 1.25–1.34, 1.76–1.85, 2.48–2.57, and 3.31–3.42. The values of ΔE_N and ΔE_D depend upon pH by a significant amount, and values appropriate to each individual pH were used for calculation of equilibrium constants. The lines drawn are for pH 2.55. The circumscribed K's shown in the figure denote initial values of the spectral intensity from extrapolation of kinetic plots (Figure 1) to zero time. The values shown all come from kinetic runs in the range of pH 2.49–2.65. ΔE has the units of (g dl)$^{-1}$ cm^{-1}.

and the results can therefore be used to calculate the equilibrium constant

$$K_D = \frac{y - y_N}{y_D - y} \tag{4}$$

as a function of pH and GuHCl concentration.

The Dependence of K_D on pH. We have followed the transition by measuring the optical rotation at 320 mμ and by measuring the change in difference spectral intensity at 301 mμ. It will be shown later that both methods lead to identical values of K_D at any set of conditions, as indeed they must if the transition involves only two states.

Figure 2 shows the results of optical rotation measurements as a function of GuHCl concentration at different pH values. For convenience, solutions along any transition curve were prepared so as to be equimolar in HCl, and since the activity coefficient of hydrogen ion increases with increasing GuHCl concentration (Nozaki and Tanford, 1967), the pH always decreased along the curve. The pH value by which each curve is labeled is the approximate value near the center of the transition. The actual pH of measurement varied considerably from the nominal values, *e.g.*, for the curve labeled pH 1.8, the pH was 2.00 in the absence of GuHCl, and 1.20 in 6 M GuHCl. The range of pH encompassed by the transition zone was much narrower, however, *e.g.*, 1.85–1.72 for the curve labeled pH 1.8. The pH ranges covered by the transition zones are shown in the caption to the figure. Measured pH values for each individual point were used for subsequent calculations.

Figure 3 shows the results of difference spectral intensity measurements as a function of GuHCl concentration at different pH values. The pH values in the figure again correspond approximately to the center of each transition zone, and differ somewhat from the actual pH values for individual points, as indicated in the figure caption.

It is noted in both figures that the denaturation transition shifts to lower GuHCl concentrations as the pH is decreased. It must therefore be possible to induce denaturation at constant GuHCl concentration by lowering the pH. The results of following the transition in this way by measuring the difference spectral intensity at 301 mμ are shown in Figure 4.

In Figure 5 the results at four concentrations of GuHCl are shown replotted in terms of log K_D as a function of pH.

As has been demonstrated by Wyman (1964), pH dependence of any equilibrium constant requires that there be a difference in the number of bound protons between the two states in equilibrium. The quantitative relationship applicable to the present data, which must hold rigorously true without any external assumption, is

$$\left(\frac{\partial \ln K_D}{\partial \ln a_H}\right)_{a_{GuHCl}} = \Delta\bar{\nu}_H = \bar{\nu}_{H,D} - \bar{\nu}_{H,N} \tag{5}$$

The left-hand side of this equation represents the slope of any of the curves of Figure 5 at a given pH, and $\bar{\nu}_{H,D}$ and $\bar{\nu}_{H,N}$ represent the number of bound protons at the same pH, in the denatured and native states, respectively. The parameters $\bar{\nu}_{H,D}$ and $\bar{\nu}_{H,N}$, as a function of pH, are simply the hydrogen ion titration curves of the denatured and native proteins.

The number of parameters required for a rigorous description of the titration curves of either the native or the denatured protein is very large. Moreover, all of these parameters are likely to differ between the native and denatured states.

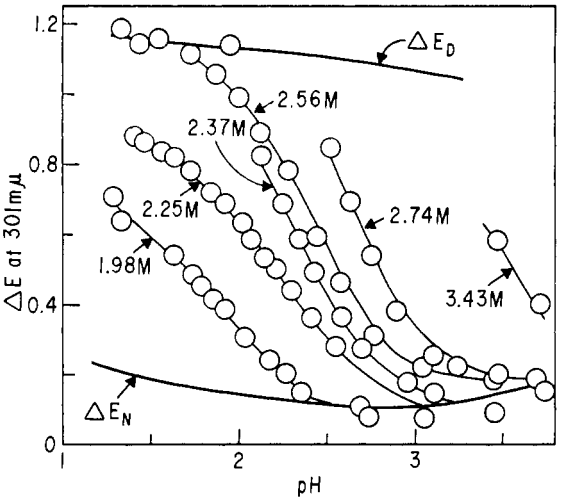

FIGURE 4: The effect of pH on difference spectral intensity at 301 mμ, at different concentrations of GuHCl. The values of ΔE_N and ΔE_D vary considerably with GuHCl concentration, and this was taken into account in calculating equilibrium constants. The lines shown correspond to 2.25 M GuHCl. ΔE has the units of $(g/dl)^{-1}$ cm^{-1}.

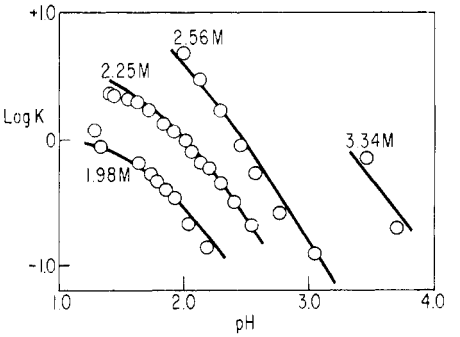

FIGURE 5: The effect of pH on log K_D at different concentrations of GuHCl. The lines drawn are theoretical, and correspond to eq 8 with $pK_{1,N} = pK_{2,N} = 1.92$, $pK_{1,D} = 3.5$, and $pK_{2,D} = 4.4$.

An equation that provides a complete description of $\Delta\bar{\nu}_H$ in terms of the properties of individual titratable groups will thus be exceedingly complex, and will contain many more terms than can conceivably be extracted from the experimental data. The customary procedure to arrive at a realistic representation of $\Delta\bar{\nu}_H$ is to assume that the difference between $\bar{\nu}_{H,D}$ and $\bar{\nu}_{H,N}$ is *predominantly* determined by differences in the titration behavior of a very small number of groups. (For example, the difference between the titration curves of oxy- and deoxyhemoglobin, which gives rise to the Bohr effect for the effect of pH on the oxygenation equilibrium, can be described as a good approximation in terms of pK differences of just two titratable groups per heme.) If we follow that procedure here, we note that the maximum value of $\Delta\bar{\nu}_H$ derived from the data of Figure 5 is approximately 2. We have accordingly attempted to describe the pH-dependence of $\Delta\bar{\nu}_H$ (in the range of pH covered by the data of Figure 5) in terms of only two groups whose pK's differ significantly in the native and denatured states. With the assumption that these groups titrate independently of each other, which, in view of the very high ionic strength of the solutions to which the equation is to apply, is a reasonable assumption for both native and denatured states, the appropriate equation for $\Delta\bar{\nu}_H$ becomes

$$\Delta\bar{\nu}_H = \frac{a_H}{a_H + K_{1,D}} + \frac{a_H}{a_H + K_{2,D}} - \frac{a_H}{a_H + K_{1,N}} - \frac{a_H}{a_H + K_{2,N}} \quad (6)$$

where $K_{i,D}$ and $K_{i,N}$ represent the dissociation constants of the groups in the denatured and native states, respectively.

One further simplification results from the fact that eq 6 is intended to apply only between 1.5 and 4.0 M GuHCl, and that the same unique states N and D are involved throughout. The dependence of the dissociation constants upon GuHCl concentration should be completely negligible within the approximation with which the equation is applicable at all (Nozaki and Tanford, 1967). Thus eq 6, with the same values of the dissociation constants, should apply at any concentration of GuHCl, from which it follows that K_D can be written as a product of two functions, one depending upon a_H only, the other on a_{GuHCl} only, *i.e.*

$$K_D = K_D^0 F(a_H) f(a_{GuHCl}) \quad (7)$$

where K_D^0 is a constant, independent of pH and GuHCl activity. Formally, it represents the value of K_D when $F(a_H)$ and $f(a_{GuHCl})$ are equal to unity. This parameter will be discussed in paper II of this series.

Substituting eq 6 into eq 5 and integrating, we get

$$F(a_H) = \frac{(1 + K_{1,D}/a_H)(1 + K_{2,D}/a_H)}{(1 + K_{1,N}/a_H)(1 + K_{2,N}/a_H)} \quad (8)$$

The dissociation constants $K_{1,D}$ and $K_{2,D}$ that occur in eq 6 and 8 are dissociation constants for a randomly coiled protein in a solution of high ionic strength and therefore represent essentially the *intrinsic* dissociation constants of the groups involved. The values assigned should be appropriate for solutions containing a high concentration of GuHCl, *i.e.*, the pK's will be slightly smaller than corresponding pK's in dilute aqueous salt solutions (Nozaki and Tanford, 1967).

To fit the data of Figure 5, it is necessary to assign very low values to $pK_{1,N}$ and $pK_{2,N}$. This probably implies that the two groups can be identified as carboxyl groups, and that $pK_{1,D}$ and $pK_{2,D}$ should have values of 3.5, 3.9, or 4.4, depending upon whether the group concerned is the terminal α-COOH group, an aspartyl group, or a glutamyl group. The choice among these values has relatively little influence on the values of $pK_{1,N}$ and $pK_{2,N}$ determined from the experimental results. The best statistical fit to the data is obtained with $pK_{1,N} \simeq pK_{2,N} \simeq 1.92$, regardless of the choice of $pK_{1,D}$ and $pK_{2,D}$.

Examination of the model structure of lysozyme, based on the X-ray diffraction studies of Blake *et al.* (1965), with due regard for the uncertainties inherent in the model, does not permit a unique choice of two carboxyl groups with abnormally low pK, as will be brought out in the Discussion section. The groups most likely to be involved, on the basis of the model, are the terminal α-COOH group and a glutamyl

group. This would mean that $pK_{1,D} = 3.5$ and $pK_{2,D} = 4.4$. The curves of Figure 5 are in fact theoretical curves, according to eq 8, with these values for the pK's of the denatured protein, and with $pK_{1,N} = pK_{2,N} = 1.92$. It is seen that they give a satisfactory description of the pH dependence at all of the GuHCl concentrations.

The agreement between theory and experiment cannot be significantly improved by increasing the number of titratable groups (with pK's below 4) involved in the reaction. If we allow for participation by an aspartyl group for example, *i.e.*, if we replace eq 8 by

$$F(a_H) = \frac{(1 + K_{1,D}/a_H)(1 + K_{2,D}/a_H)(1 + K_{3,D}/a_H)}{(1 + K_{1,N}/a_H)(1 + K_{2,N}/a_H)(1 + K_{3,N}/a_H)} \quad (9)$$

with $pK_{3,D} = 3.9$, we find that the value for $pK_{3,N}$ cannot be significantly lower than 3.9. Because our data are for practical purposes confined to the range of pH 1–3.5, groups with pK $\geq$ 4 have little influence, *i.e.*, our results cannot provide information about groups with higher pK that might differ significantly in the two states of the protein.

Although the identity of the two abnormal groups of the native protein as carboxyl groups cannot be established on the basis of the data of this paper alone, it is strongly supported by combining the results of this paper with those of our earlier study at pH 5.5 (Tanford *et al.*, 1966). Inclusion of higher pH data allows us to obtain information about groups with pK > 4. For example, curves 1 and 2 of Figure 6 both represent eq 8 with $pK_{1,N} = pK_{2,N} = 1.92$. Curve 1 represents the values of $pK_{1,D}$ and $pK_{2,D}$ used earlier, but in curve 2 it has been assumed that one of the abnormal groups is an imidazole group instead of a carboxyl group by placing $pK_{2,D} = 6.5$. The two curves are the same below pH 4, but differ above that pH, because $\Delta\bar{\nu}_H$ (eq 6) cannot become zero, *i.e.*, curve 2 cannot level off, until the pH exceeds the pK's of the abnormal groups in the denatured state. Comparison of the previous data with those of this paper shows that curve 2 is not in accord with the experimental results. At 3.85 M GuHCl and 25°, for example,[3] log K_D at pH 3.4 (from Figures 2 and 3) is 0.35; at pH 5.5 at the same concentration of GuHCl and the same temperature, log $K_D = -0.45$. Thus Δ log K_D between the two pH values is -0.8, whereas curve 2 predicts a difference of -2.4.

Actually even curve 1 does not provide an accurate description of the pH dependence above pH 4: it predicts a Δ log K_D of approximately -1.2 between pH 3.4 and 5.5, whereas the experimental difference is approximately -0.8. To improve the agreement between theory and experiment it is necessary to introduce additional groups, with abnormal pK's in the native state. In particular, at least one group must have an abnormally *high* pK in the native state, to account for experimental log K_D values lying *above* curve 1. The presence of the requisite groups is actually already established on the basis of other work, to be discussed in the Discussion section. Curve 3, based on the results to be considered there, leads to a difference in log K_D of -0.74, between pH 3.4 and 5.5, in agreement with the experimental difference of -0.8.

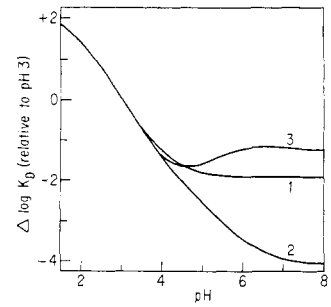

FIGURE 6: The calculated effect of pH on log K_D. Curve 1 represents eq 8, with the parameters used in Figure 5. Curve 2 represents the same equation, but with $pK_{2,D} = 6.5$ in place of 4.4. Curve 3 represents eq 10, with the pK values given in the text.

Discussion

It has been established on the basis of kinetic studies of denaturation and renaturation in GuHCl solutions from concentrations of 1.9–5 M and pH values of 1.3–5.5 at 25° that only native and random coil states contribute significantly to the equilibrium under these conditions. This means that the effect of pH within this pH range is to be ascribed entirely to the effect of pH on a single equilibrium constant, K_D, for the reaction N $\rightleftharpoons$ D, and is therefore rigorously related to the difference in titration curves between the two conformations N and D. It has been shown that the effect between pH 1 and 3.5 is due primarily to two (and *only* two) acidic or basic groups with pK values of about 1.9 in the native state. It was assumed that these are carboxyl groups, and this assumption was confirmed by making use of results obtained at higher pH. In addition, the results at higher pH require the introduction of one or more additional titratable groups with abnormal pK in the native state, and at least one group with an abnormally high pK.

The factor most likely to influence the pK's of titratable groups of proteins is coulombic interaction between charged sites. We have inspected the model of lysozyme derived from the X-ray diffraction studies of Blake *et al.* (1965), to locate possible sites for such interactions.[4] In interpreting this model we must keep in mind that we are dealing with the molecular properties of the protein at very high ionic strength, and that long-range electrostatic forces will therefore be effectively eliminated even in the native state. Short-range electrostatic interactions, between groups so close to each other that the ions of the added electrolyte cannot penetrate between them, will, however persist. In native lysozyme, such interactions could affect the terminal α-COOH group, which the model shows to be only 3.5 Å from the ϵ-amino group of Lys-13,

[3] An extension of Figure 5 to include results at pH 5.5 is not possible because the data from the earlier study were obtained at higher GuHCl concentrations than the data of the present paper given in Figure 5. Comparable results at the same concentration of GuHCl can be obtained only if we use the data of Figures 2 and 3 near the completion of the transition zone. The previous data were also obtained at a different temperature (30°), and the value of log K_D at 25° cited in the text was obtained by applying a correction for the temperature difference based on temperature-dependence measurements to be reported in a later paper.

[4] We wish to express our appreciation to Professor D. C. Phillips for making preliminary values of the atomic coordinates of the structure available to us.

and the side-chain carboxyl group of Glu-7, which the model shows to be about 4.5 Å from the ϵ-amino group of the amino-terminal lysine residue. The charge separations for these groups are sufficiently small to lead to pK values of 2–3 pK units below intrinsic pK's. (It is for this reason that the values of $pK_{1,D}$ and $pK_{2,D}$ in eq 8 were chosen to be appropriate for an α-COOH and a glutamyl group.)

Two other possibilities are the side-chain carboxyl groups of Asp-48 and -103, each of which is shown by the model to be about 6.5 Å from the charged group of an arginine side chain. Since the precise locations of the charged groups at the surface of the protein molecule represent the least accurate part of a structure determined by X-ray crystallography, these groups cannot be eliminated from consideration.

Another possible carboxyl group with abnormally low pK is Asp-52, which the model structure shows to be hydrogen bonded to two vicinal amide groups. There is no compelling argument to suggest that hydrogen bonding to amide groups instead of water molecules should have a large effect on the pK, but systematic knowledge of such effects is lacking, and the possibility must be kept in mind.

Abnormal pK values in native proteins can also result if a titratable group is located in an environment consisting largely of hydrophobic parts of the molecule: the charged form of an acidic group would be destabilized by such an environment. For a carboxyl group, therefore, the pK would be abnormally high. As has been discussed by Rupley *et al.* (1967), Glu-35 satisfies these criteria, and is thus likely to represent the group with high pK in the native state that is required to account for the experimental results. Inspection of the model does not indicate that other carboxyl groups should be subject to a similar effect.

Additional information can be obtained from the studies of Rupley *et al.* (1967) and Dahlquist and Raftery (1968) of the pH dependence of the binding of saccharides to lysozyme. The results of these studies implicate the involvement of three carboxyl groups,[5] one of which has a pK of about 6.1 in the native protein. As has already been mentioned, it has been reasonably well established that this must be Glu-35. One of the other two groups has an essentially normal pK, but the third group has a pK of 1.8–2.0 in the free native enzyme. This group is of particular importance for the present analysis, for it indicates that one of the two low pK carboxyl groups required by our data must be located in the binding site for saccharides. Neither the α-COOH group nor Glu-7 fulfil this requirement: examination of the model indicates that the only carboxyl groups near the saccharide binding site (apart from Glu-35) are Asp-52, -101, and -103. Two of these (residues 52 and 103) have already been judged as possibly having a low pK in the native state on the basis of examination of the model structure, but Asp-101 should have a normal pK on this basis. Dahlquist and Raftery (1968) have also presented evidence, on the basis of comparative studies of the binding of monosaccharides and trisaccharides to lysozyme, that the ligand-linked aspartyl group with a normal pK should be Asp-101.

Thus either Asp-52 or -103 may be involved. It is not important to make a choice here: it suffices to note that one of the groups with low pK must on the basis of the binding studies be an aspartyl residue. This means that one of the COOH groups judged to have a low pK on the basis of the model structure cannot be located (in dilute GuHCl solution) in the environment given by the model coordinates. We believe it is more likely that this is the α-COOH group: the side chain of Lys-13 may extend into the surrounding solvent when the protein molecule is in solution.

One additional study of interest in connection with our analysis is the nmr study of native lysozyme by Meadows *et al.* (1967), which shows that the protein's lone histidine residue has a pK of 5.8 in the native state, as compared with the normal intrinsic pK of 6.4. While this is a relatively small anomaly it should be included to account for the pH dependence of denaturation in the neutral pH region.

The final conclusion is therefore that four groups are expected to contribute to the pH dependence of denaturation at acid and neutral pH, these being Asp-52 or -103, Glu-7, and -35, and His-15. The equation for $F(a_H)$ becomes

$$F(a_H) = \prod_{i=1}^{4}(1 + K_{i,D}/a_H) \Big/ \prod_{i=1}^{4}(1 + K_{i,N}/a_H) \qquad (10)$$

with $pK_{1,N} = pK_{2,N} = 1.9$, $pK_{3,N} = 6.1$, $pK_{4,N} = 5.8$, $pK_{1,D} = 3.9$, $pK_{2,D} = pK_{3,D} = 4.4$, and $pK_{4,D} = 6.4$. Curve 3 of Figure 6 has been drawn according to eq 10, with these parameters, and, as was indicated earlier, is consistent both with the data of this paper, and with the earlier data of Tanford *et al.* (1966).

Curve 3 also agrees with the results of Ogasahara and Hamaguchi (1967), who have measured the pH dependence of the denaturation of lysozyme by GuHCl, at a denaturant concentration of 3.84 M, between pH 3.2 and 8. Their results are in good agreement with ours. Their over-all pH dependence is essentially that given by curve 3 of Figure 6.

As an over-all check on our analysis, we have reduced all the data obtained in this paper to the same pH by use of the function $F(a_H)$. If the correct expression for $F(a_H)$ is used all points should fall on a smooth curve when plotted as a function of the concentration of GuHCl. The appropriate data are shown in Figure 7. The ordinate represents the logarithm of $K_D/F(a_H)$, which, by eq 7, represents the logarithm of the product $K_D{}^0 f(a_{Gu\cdot HCl})$. Alternatively, it may be regarded as the value of K_D at infinite hydrogen ion activity, since $F(a_H)$ is then equal to unity. All experimental data with $0.2 \leq K_D \leq 5$ are included, these being the limits within which the results have the greatest experimental precision. Both results based on optical rotation experiments and results based on difference spectral data are included. Three of the experimental points are taken from our earlier study at pH 5.5, after correction to 25°. The figure shows that the experimental points from optical rotation and difference spectral data are, within experimental error, superimposable on the same curve. This is a necessary consequence of the already established fact that the transition is a two-state transition without stable intermediates. It also demonstrates that all results fall on a single curve, regardless of the pH of measure-

[5] Lin and Koshland (1969) have shown that glutamic acid 35 and aspartic acid 52 are the only carboxyl groups *essential* for the active site of lysozyme: activity is retained when all other carboxyl groups are esterified. This result does not contradict the conclusion that one or two additional carboxyl groups are influenced by substrate binding if present in the unesterified state.

ment, confirming both the validity of eq 7 for K_D and of eq 10 for $F(a_H)$.

It is worth noting that comparable results are obtained for the pH dependence of the thermal denaturation of lysozyme, as reported by Sophianopoulos and Weiss (1964), and for the pH dependence of the denaturation by GuHCl. The products of the two denaturation processes are different: the product of heat denaturation, though substantially disordered, retains a region of ordered cooperative structure, that can be disrupted by the addition of GuHCl, after the thermal denaturation is complete (Aune *et al.*, 1967). The pH dependence of the two processes is, however, quite similar. If one takes into consideration the fact that the thermal denaturation was carried out at relatively low ionic strength, where long-range electrostatic interactions cannot be ignored, the results suggest that the two carboxyl groups with low pK in the native protein lie in that part of the structure which becomes disordered in the thermal transition. Sophianopoulos and Weiss (1964) showed that $\Delta\bar{\nu}_H$ for the thermal transition (pH 1–3) approaches three protons per molecule. One of these protons, however, can reasonably be assigned to the effects of long-range electrostatic interactions, *i.e.*, even normal carboxyl groups will not be entirely protonated in the native state near pH 2, but protonation should be more nearly complete in the denatured state where the electrostatic interactions are substantially reduced. If short-range interactions are invoked for the other two groups, Sophianopoulos and Weiss' analysis shows that they would have to have pK's in the native state between 1.46 and 1.72. These pK's are lower than the pK of 1.92 assigned to both abnormal groups in the present paper, but again this is a reasonable difference ascribable to the fact that the abnormal groups will be subject to long-range electrostatic interactions at relatively low ionic strength, in addition to the short-range interactions that persist to high ionic strength.

It may be observed, in conclusion, that the existence of titratable groups with abnormally low pK in the native protein has been demonstrated by the titration studies of native and denatured lysozyme reported by Donovan *et al.* (1960). On the other hand, no such abnormal groups were reported as being present by Tanford and Wagner (1954). This discrepancy is probably explained by another observation of Donovan *et al.* (1960), namely, that the preparation of lysozyme used by Tanford and Wagner contained more titratable carboxyl groups than would be expected on the basis of the amino acid composition of the protein, possibly reflecting hydrolysis of some amide groups in the course of preparation of the sample. The acid end point of the titration curve of Tanford and Wagner was based on data at ionic strength 0.15, but is by no means unequivocally established by the data. In fact, if one experimental point at low pH is omitted, an extrapolation to allow for two additional carboxyl groups with pK's in the vicinity of 1.5 is entirely reasonable. The extrapolation as actually performed in the paper seemed reasonable at the time, because it led to stoichiometry in agreement with the reported amino acid analysis.

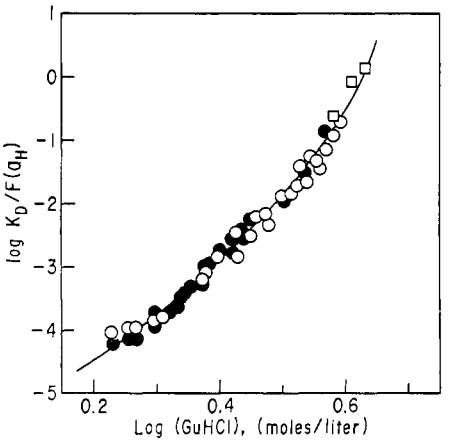

FIGURE 7: The effect of GuHCl concentration on log $[K_D/F(a_H)]$. Equation 10 was used for $F(a_H)$. Filled circles represent difference spectral measurements at 301 mμ. Open circles represent optical rotation measurements at 320 mμ. The three squares represent data of Tanford *et al.* (1966), corrected to 25°.

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