case (Malmström *et al.*, 1970) a great deal of evidence for this has been accumulated. The reaction of laccase with cyanide was extensively studied (Malkin *et al.*, 1968) and shows indeed many similarities to that of the bovine enzyme. It may then not be too farfetched to suggest also a similar functional disposition for both laccase type 2 Cu(II) and copper of the bovine enzyme. In the latter case it is tempting to postulate that this anion binding position is the very site of attachment of the proposed substrate, the superoxide anion radical. The reaction of the bovine enzyme copper with H_2O_2—which is a product of the dismutase reaction—may be taken as a *prima facie* evidence that investigations of this aspect might be revealing.

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Interpretation of Protein Titration Curves. Application to Lysozyme†

Charles Tanford* and Robert Roxby‡

ABSTRACT: A computer method has been devised for the calculation of hydrogen ion titration curves of proteins according to the theory of Tanford and Kirkwood. The theory is moderately successful in accounting for the experimental titration curve of lysozyme, but only if one of the parameters of the theory is assigned a value that differs from the value expected for it on the basis of previous studies. It is also shown that some titratable groups on lysozyme are significantly affected by nonelectrostatic interactions that are not theoretically predictable, so that *ad hoc* pK assignments have to be made for them. One other difficulty arises from the likelihood that some titratable groups of a protein molecule will have different locations in solution and in the crystalline state, so that calculations based on the structure of the crystalline protein will be subject to error. The overall conclusion is that although the major perturbation of the acidic and basic groups of proteins arises from electrostatic interactions between charged sites, the accurate prediction of pK values of individual groups is not feasible.

It is well known that the titration curves of native proteins differ substantially from the sums of the unperturbed titrations of the constituent acidic and basic groups. It has been commonly assumed that the dominant factor responsible for the difference arises from electrostatic interactions between the titratable groups when they are in their charged

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state. There is strong experimental support for this assumption in that an increase in ionic strength greatly diminishes the difference between experimental curves and those calculated on the basis of the intrinsic properties of the constituent acidic and basic groups alone (Cannan *et al.*, 1941, 1942). This support is augmented by the fact that the theoretical equation of Linderstrøm-Lang (1924) can account approximately for the phenomenon itself, and for the effect of ionic strength upon it, in several proteins. The failure of the Linderstrøm-Lang theory to account *quantitatively* for experimental results in most instances is not surprising, because its treatment of electrostatic interactions is grossly oversimplified in that it assumes all charges to be smeared uniformly over the surface of the protein molecule. A refined theoretical treatment was proposed by Tanford and Kirkwood (1957), based on the model of Kirkwood (1934) for the calculation of electrostatic interactions. This model takes cognizance of the fact that actual charges on a protein molecule exist as discrete unit charges, distributed nonuniformly at specific sites. To use this theory, the coordinates of these sites have to be known, and interaction free energies have to be calculated as the sums of terms representing the interactions of all possible pairs of sites, which creates a formidable computational problem. Only one attempt to make an actual calculation has so far been reported, this being for hemoglobin (Orttung, 1969, 1970). It was found in this case that a critical parameter in the theory, previously determined on the basis of pK measurements of small molecules, had to be altered in order to obtain agreement between theory and experiment, and this finding suggests that the Tanford–Kirkwood treatment may in fact not be valid for application to protein titration curves.

Another problem in the interpretation of the titration curves of native proteins is that, though electrostatic interactions are the most important factor perturbing the pK's of acidic and basic groups, other factors also play a role. The suggestion that other factors could account for the entire perturbation (Karush and Sonenberg, 1949) need not be taken seriously since they could not account for the observed effect of ionic strength. However, the failure of a few groups on native protein molecules, notably phenolic groups, to contribute to the titration curve at all is undoubtedly due to their being buried in a hydrophobic region (Tanford *et al.*, 1955), and this raises the obvious possibility that other titratable groups may be in a partially hydrophobic environment where access by H^+ or OH^- is possible, but the free energy of association anomalous. Hydrogen bonds between surface groups could also affect pK values. Both these possibilities are difficult to test because it is not possible to predict the magnitude of the effects even if the locations of the groups involved are known. A method for making a calculation of the effect of hydrogen bonds has been proposed (Laskowski and Scheraga, 1954), but the heats and entropies of hydrogen-bond formation that it employs ignore interactions with water and are grossly unrealistic.

The only aspect of the problem of protein titration curves that can be considered free of uncertainty is the assignment of intrinsic (*i.e.*, unperturbed) pK values to the constituent groups. These pK's are derived on the basis of model compounds, but their applicability to the constituent groups of proteins has been tested by the ability to make quantitative predictions of experimental titration curves of proteins in concentrated guanidine hydrochloride (Nozaki and Tanford, 1967a; Roxby and Tanford, 1971). Proteins in this solvent are in a randomly coiled state and subject to no significant noncovalent interactions of any kind, and the calculation depends only on knowledge of the intrinsic pK values.

The purpose of this paper will be to consider these various problems in the interpretation of hydrogen ion titration curves in relation to the experimental titration curve of lysozyme. A new and simple procedure for making calculations on the basis of the Tanford–Kirkwood theory will be described. It will make use of published intrinsic pK values for the constituent groups, and positional coordinates based on the three-dimensional structure in the crystalline state (Blake *et al.*, 1967). Lysozyme was chosen for this study because its native conformation is more stable toward pH changes at room temperature than that of most other proteins, so that the entire titration curve can be taken to be that of the protein in a single conformation.[1] This conformational stability also increases the confidence with which one can make the assumption that the locations of titratable groups on the native molecule in solution are the same as in the crystalline state, an assumption which has to be made if a calculation is to be attempted. An additional advantage to the use of lysozyme is the considerable amount of work that has been done on the pH dependence of enzymatic and spectral properties of the protein, on the basis of which the titration characteristics of certain individual groups may be inferred.

Materials and Methods

Preparative procedures and measurement of titration curves by the continuous technique were carried out as described previously (Roxby and Tanford, 1971). To achieve greater precision near the ends of the titration curves the batchwise procedure described by Tanford (1955) was used.

Experimental Results

The titration curves of lysozyme at two ionic strengths were redetermined because recent data from several sources (Sophianopoulos and Weiss, 1964; Rupley *et al.*, 1967; Dahlquist and Raftery, 1968; Aune and Tanford, 1969) indicate the presence of one or more carboxyl groups with an extremely low pK. The presence of these groups was not detected in previous titration studies (Tanford and Wagner, 1954), though strong indirect evidence for their existence was provided by Donovan *et al.* (1960). The experimental results are shown in Figure 1, and the titration curve of the same sample of protein in 6 M guanidine hydrochloride, reported previously (Roxby and Tanford, 1971), is included for comparison. As the subsequent calculations will show the results do require that one carboxyl group (but only one) be assigned an anomalously low pK.

It may be noted that the failure to observe the anomalous carboxyl group in previous studies is chiefly the result of variability in the number of free carboxyl groups in different lysozyme samples. The previous studies did not extend experimentally to as low a pH as the present studies. The acid end point of the titration was determined by extrapolation from data at somewhat higher pH, and, in the case of the study by Tanford and Wagner (1954) the total number of carboxyl

[1] Temperature-jump kinetic studies (Owen *et al.*, 1969) have presented evidence for a measurably slow reaction involving proton exchange, taking place around pH 7. If this represents a conformational change it must be a very localized one because optical rotation and other properties directly related to conformation do not undergo alteration at this pH.

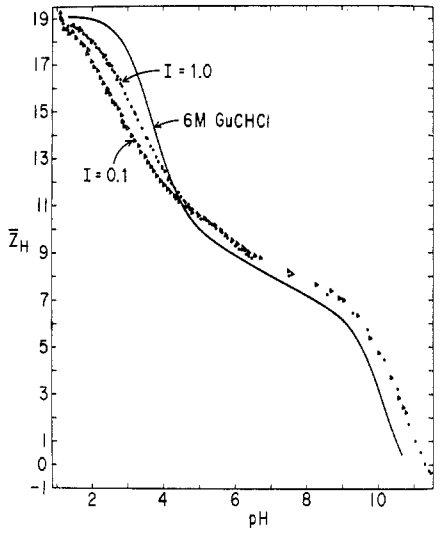

FIGURE 1: The titration curve of native lysozyme in KCl solutions of ionic strength 0.1 and 1.0, at 25°. The titration curve of the denatured protein in 6 M guanidine hydrochloride is included for comparison.

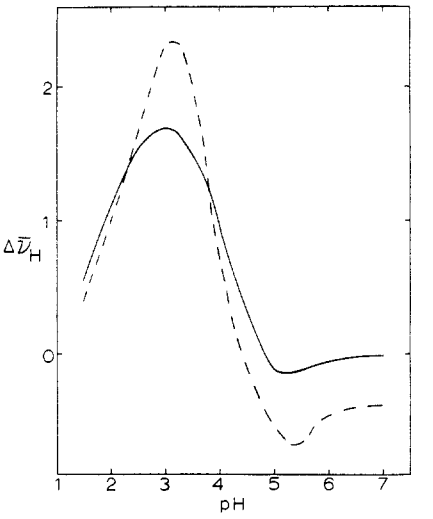

FIGURE 2: Differences in protons bound, $\Delta\bar{\nu}_H$, between unfolded and native lysozyme as a function of pH. Solid line determined from the pH dependence of denaturation, broken line from the direct titration curves.

groups thus determined proved to be equal to the number based on the amino acid sequence subsequently reported by Canfield (1963). In the case of the study by Donovan *et al.* (1960) the observed number was even larger. Thus no reason existed for attempting to locate an additional carboxyl group by more careful measurements at extreme pH. Assuming that the anomalous group was present in these preparations, it would appear that the preparations previously used had more free COOH groups than predicted by the amino acid sequence. Titration of the present sample in 6 M guanidine hydrochloride showed that it has one *fewer* free COOH group than predicted by the sequence.

Figure 2 shows a plot of the difference in proton binding between native lysozyme (in 1 M KCl) and the denatured protein (in 6 M guanidine hydrochloride) as a function of pH. The figure also includes the quantity $\Delta\bar{Z}_H$ that one obtains by application of the theory of linked functions (Wyman, 1964) to the effect of pH on the equilibrium constant K_D for the denaturation of lysozyme in guanidine hydrochloride solutions (Aune and Tanford, 1969) by the relation

$$\frac{\partial \ln K_D}{\partial \ln a_{H^+}} = \Delta\bar{Z}_H \qquad (1)$$

$\Delta\bar{Z}_H$ as calculated by this equation represents the difference in proton binding between native and denatured protein molecules in equilibrium with each other in the same solution: the data apply to solutions containing about 3 M guanidine hydrochloride. It is seen that these two quite different ways of obtaining the difference between the titration curves of native and denatured lysozyme are in excellent agreement. The difference between them is in the expected direction since the titration curve of native protein in a 3 M salt solution should be somewhat closer to that of the denatured protein than the titration curve in 1 M salt.

Inspection of the results in Figures 1 and 2 immediately reveals that the titration behavior of lysozyme differs greatly from that predicted by the Linderstrøm-Lang theory. The titration curve in guanidine hydrochloride may be taken as a model for the unperturbed titration curve in KCl solutions, since intrinsic pK's in the two solvents differ little (Nozaki and Tanford, 1967b). The effect of the perturbing interactions is thus seen to increase the number of bound protons in the native state from pH 4.5 upward, in spite of the fact that the net charge of the protein is positive throughout the range in which the data were taken. This can only mean, contrary to expectation, that there are groups which in the native state have higher pK's than in the unfolded state despite the presence of a considerable excess of positive charges. Moreover, between pH 4.5 and 9, there is essentially no effect of ionic strength on the titration of the native protein, indicating that the principal perturbation affecting this part of the titration curve does not arise from electrostatic interaction.

On the basis of a number of different studies of pH-dependent properties of lysozyme it has been determined that a group almost certainly identified as glutamic acid residue 35 has an anomalous pK of 6.3 (Rupley *et al.*, 1967; Lin and Koshland, 1969). This is nearly two pK units above the intrinsic pK for a glutamyl carboxyl group of 4.5 (Nozaki and Tanford, 1967c). This anomaly cannot be the result of electrostatic interactions with other charged groups, as the crystal structure shows no closely placed anionic groups. It has been suggested (Blake *et al.*, 1967) that the active-site cleft in which this residue is located contains a sufficient concentration of nonpolar side chains to stabilize the uncharged form of this COOH group, and that this is responsible for the high pK. Regardless of the explanation, the presence of this anomalous group is able to account for much of the observed titration anomaly. It cannot, however, account for all of it, for if this were the only source of anomaly the titration curves of native and denatured would superimpose again at about pH 7.5, whereas in fact they remain separated by a fraction of one group. This suggests that the native protein contains a second group with an anomalously high pK, and it is very probable that this is the terminal α-amino group, because, unless there are strong nonelectrostatic perturbations not yet recognized, it is this group that should make the major contribution to the total titration curve in this pH range. (The titration curve is very flat here and, in the native protein, $\bar{Z}_H$ changes by only 1.0 between pH 7.5 and 9.0.)

Method of Computation

The model of Kirkwood (1934) represents the protein molecule as a spherical cavity of radius b with low internal dielectric constant, embedded in a solvent of high dielectric constant. Mobile ions in the solvent may approach to within a distance a from the center of the cavity, $a - b$ being a measure of the average ionic radius. The free energy of interaction (W_{ij}) between two sites i and j on the protein molecule is given by

$$W_{ij} = \frac{\epsilon^2 z_i z_j}{2b}(A_{ij} - B_{ij}) - \frac{\epsilon^2 z_i z_j}{2a} C_{ij} \qquad (2)$$

where ϵ is the electronic charge and z_i and z_j are the charges at sites i and j in units of ϵ, *i.e.*, for protein side chains they may take on values of $+1$, -1, or 0. A_{ij}, B_{ij}, and C_{ij} are complex functions, expression for which are given by Tanford and Kirkwood (1957). $A_{ij} - B_{ij}$ represents the interaction energy at zero ionic strength and C_{ij} the modification of this energy by mobile ions at nonzero ionic strength. It has been shown (Tanford, 1957) that the crucial parameters affecting the value of W_{ij} are the distance r_{ij} between the sites and the depths d_i and d_j below the cavity surface at which the sites are located. The size of the cavity (as expressed by a and b) is less important.

When eq 2 was used to estimate the effects of charge interactions on small molecules with known charge separations (Tanford, 1957), it was found that the theory could account for experimental pK differences with remarkable accuracy, but only if a fixed value of 1.0 Å was assigned to the depth of any charge within the cavity. Physically this suggests that all charges seek to be as close to the high dielectric solvent as possible and the fact that d_i and d_j are not equal to zero under these conditions may be interpreted as a formal consequence of two features of the theoretical model: (1) the treatment of charges as dimensionless points, and (2) the treatment of the solvent as a continuum. In actuality the charges are located on atoms of finite size and the solvent consists of molecules of finite size, so that the closeness of approach of solvent to charge is necessarily limited. The distance of 1 Å may thus be taken as a formal measure of this limit.

The effect of charge interactions on the pK of a particular group i is determined by the interaction of all other charges with the charged form of group i, and the effect of a positive charge at site j is always to decrease pK_i regardless of the charge type of group i itself. For a particular constellation of charges, pK_i may thus be related to its intrinsic pK (p$K_{\text{int,i}}$) by the relation

$$\text{p}K_i = \text{p}K_{\text{int,i}} + \sum_{j \neq i} \Delta\text{p}K_{ij} \qquad (3)$$

where

$$\Delta\text{p}K_{ij} = -W_{ij}/2.303 z_i kT \qquad (4)$$

and the sum in eq 3 extends over all sites on the molecule other than site i itself.

The results obtained with small molecules suggest that the depth parameters d_i and d_j may be treated as a single constant d. For a particular protein with fixed dimensions, at a given ionic strength, W_{ij} as calculated by eq 2 then becomes a unique function of r_{ij} and the product $z_i z_j$. Thus we may replace $\Delta\text{p}K_{ij}$ in eq 3 by

$$\Delta\text{p}K_{ij} = -z_j|\Delta\text{p}K(r_{ij})| \qquad (5)$$

where $|\Delta\text{p}K(r_{ij})|$ is a unique function of r_{ij} alone. A computer program was used to generate values for this parameter at appropriate intervals of r_{ij} over the full possible range and the points were fitted to a convenient algebraic expression, which could then be used in the procedure described below. Figure 3 shows an example of a plot of $|\Delta\text{p}K|$ *vs.* r_{ij} for a particular choice of the pertinent fixed variables.

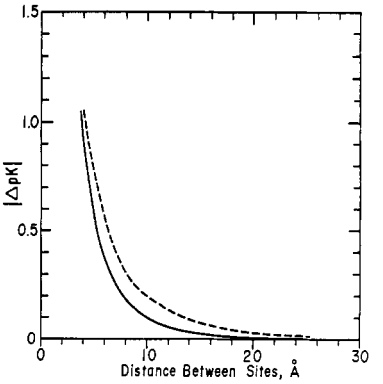

FIGURE 3: Energy functions used in titration curve calculations, calculated from Tanford–Kirkwood theory with d = 0.4 Å, D_i = 4.0, and other parameters as described in text. Dashed line, I = 0.1 m; solid line, I = 1.0 m.

Computational Algorithm. A simple and efficient iterative procedure for calculating the effective pK values for all constituent groups at a given pH was devised. It was first assumed that the equilibrium between acidic and basic forms of each titratable group is governed by its intrinsic pK. This leads at once to an initial value for the average charge $\bar{z}_j$ at each site. These values were inserted in eq 5 to generate a set of $\Delta\text{p}K_{ij}$ values for site i, which were inserted in eq 3 to yield an effective pK for group i. The process was repeated for all constituent groups. The new set of pK values led to a new set of values for the average charge $\bar{z}_j$ at each site and these in turn were used to obtain an improved set of effective pK values. It was found that six iterations of this process were usually sufficient for convergence of the pK's to values that were constant to better than 0.1 pK unit. These constant values were then used to calculate the net number of bound protons. The overall calculation at each pH requires a 5-sec computer time.

It should be emphasized that the pK values employed in this calculation apply only to one particular pH and are used only to calculate the average degree of dissociation of each group at that pH. They are not intended as representative of the overall course of titration of the groups as a function of pH. On the contrary, the pK used for a given group would in general be expected to alter as the pH is changed.

Specific Assumptions and the Choice of Structural Parameters. Values for some of the structural parameters were chosen as follows and were invariant throughout. The radius, b, was taken to be that of a sphere of the same mass and density, including 20–30% hydration, as that of lysozyme and calculated to be 17.5 Å. The ionic exclusion radius was taken to be 20.0 Å. The external dielectric constant was set at 78.5, and the temperature at 298°K. An internal dielectric

TABLE I: Calculated pK's for Groups Dissociating in the Acid Region.[a]

Residue and Position in Sequence		Calculated pK			
		I = 1.0 m		I = 0.1 m	
		pH 2	pH 6	pH 2	pH 6
Glu	7	3.0	3.2	2.4	2.7
His	15	5.3	5.8	4.8	5.6
Asp	18	3.3	3.4	2.9	3.2
Asp	48	3.3	3.6	2.9	3.4
Asp	52	3.7	4.1	3.3	4.0
Asp	66	3.3	3.5	2.8	3.2
Asp	87	3.1	3.5	2.5	3.2
Asp	101	3.5	3.8	3.0	3.6
Asp	103	3.6	3.9	3.2	3.7
Asp	119	3.7	3.7	3.3	3.4
α-COOH	129	1.5	1.7	0.8	1.2

[a] The intrinsic pK's are 3.6 for the α-COOH group, 4.0 for Asp, 4.5 for Glu, and 6.4 for His.

constant of 4.0 was usually employed. This is the dielectric constant of liquid acetamide and is more appropriate for the interior of a protein molecule than the value of 2.0 (representative of liquid hydrocarbon) often used in calculations based on the Kirkwood model. A similar choice was made by Orttung (1969, 1970). Calculations were made with several choices of the depth parameter d, which, as previously stated, is taken to have the same value for all charged sites. Charge separations r_{ij} were based on the coordinates obtained from crystal structure (Blake *et al.*, 1967) which were kindly supplied to us by Dr. D. C. Phillips. The intrinsic pK values used were those listed by Nozaki and Tanford (1967b). The possible effect of ionic strength on these parameters was neglected.

A complication is introduced by the fact that our sample of lysozyme had one fewer free carboxyl group than predicted by the amino acid sequence (Roxby and Tanford, 1971), and there is no way to identify the missing group. The procedure used was to make an initial calculation on the basis of all carboxyl groups expected on the basis of the amino acid sequence. Two of these have highly anomalous pK values, as will be discussed, and there is evidence that both must be present in all lysozyme samples, regardless of total carboxyl group content. The expected titration for our sample was obtained by subtracting the contribution of an average of the remaining groups from the initial result.

Calculated Results

It became evident early in the calculation that use of a value of 1.0 Å for the depth parameter d, as suggested by similar calculations for small molecules (Tanford, 1957), leads to unacceptably large values for $|\Delta pK|$. In order to obtain any agreement at all between experimental and calculated values this parameter had to be reduced to a value between 0.2 and 0.5 Å. The actual optimal value depends on the choice of a value for the internal dielectric constant, but no reasonable value will permit $d \simeq 1.0$ Å. Orttung has made a similar observation with respect to calculation of the titration characteristics of hemoglobin, and has suggested that for that protein $d = 0$ may be the best value (Orttung, 1970). For calculations in this paper we have used $d = 0.4$ Å, giving rise to the $|\Delta pK|$ values in Figure 3.

As previously stated, there is no possibility of accounting for a pK of 6.3 for glutamic acid residue 35 on the basis of interaction between charged groups, and the perturbation of this group must therefore be assigned to some other factor, such as the presence of predominantly hydrophobic groups in the vicinity of its location in the active-site cleft (Blake *et al.*, 1967). The same situation exists with respect to the α-amino group. It was noted earlier that this group must have an anomalously high effective pK to account for the location of the experimental titration curve between pH 7 and 9. This is confirmed when detailed calculations are made and the actual pK assigned has to be about 8.5, *i.e.*, 0.7 pK unit above the intrinsic pK. There are no nearby anionic sites to account for this effect, but the group is located in an indentation of the protein surface and hydrogen bonded to the hydroxyl group of threonine residue 40. Thus there are interactions that could account for the observed pK, though there is no *a priori* reason why a hydrogen bond should preferentially stabilize the cationic form of the amino group. Finally, the three tyrosyl residues must be added to this list of acidic groups the pK of which cannot be explained on the basis of coulombic interactions: the spectrophotometric titration of lysozyme is consistent with a pK of about 10.8 for all three of the groups (Donovan *et al.*, 1961).

Apart from these assigned pK's and the empirical value of $d = 0.4$ Å mentioned earlier, no *ad hoc* assumptions were made. The literature values for intrinsic pK's were used and positional coordinates were fixed on the basis of the crystal structure. Table I illustrates the order of magnitude of the computed effects of charge interactions on pK values. The effects are seen to be significantly larger at ionic strength 0.1 than at ionic strength 1.0; in fact, the difference between the titration curves at the two ionic strengths seen in Figure 1 is very well accounted for. The magnitude of the shift in pK is seen to vary from one group to another. The terminal α-COOH group, which in the crystal structure is hydrogen bonded to the amino group of lysine residue 13, is seen to have an especially low pK, and this agrees at least superficially with the earlier evidence for the existence of one or more carboxyl groups with exceptionally low pK values. The overall calculated titration curve at ionic strength 1.0 is shown in Figure 4 in comparison with the experimental data, and the agreement is seen to be good, though not perfect. Equally good agreement is obtained between calculated and observed results at ionic strength 0.1. The dashed line in Figure 4 shows that it was indeed necessary to assign pK values for the α-amino group and the three tyrosyl residues before beginning the calculation based on electrostatic interactions alone.

The agreement with experimental data extends beyond the overall titration curves. The external evidence for a carboxyl group with very low pK has already been noted. Another group on which there is other evidence is the single histidine residue. The pK of the imidazole moiety of this residue has been measured by Meadows *et al.* (1967) on the basis of the pH dependence of the chemical shift of one of the protons. This measurement was carried out in D_2O. Corrections for the isotope effect on the pK (Li *et al.*, 1961; Bradbury and Scheraga, 1966) and for the solvent effect on the glass electrode (Glasoe and Long, 1960) nearly offset one another, so that the pK in water should be the same as the pK of 5.8 measured in D_2O or slightly less. The calculated pK (at pH 6) in Table I is 5.6 at ionic strength 0.1 and 5.8 at ionic strength 1.0.

In regard to the carboxyl group with very low pK, Aune and Tanford (1969) showed that the simplest way to account for the difference curve of Figure 2 in the acid region was to assume that there are two carboxyl groups with very low pK in the native state, rather than one. Their data can however be described equally well by one such group together with smaller contributions to $\Delta\bar{Z}_H$ by a large number of groups undergoing smaller shifts in pK on denaturation, *i.e.*, there is no contradiction between the results. The more complete data presented in this paper exclude the possibility of a second group with very low pK with reasonable certainty, since all of the carboxyl groups of Table I necessarily contribute to $\Delta\bar{Z}_H$.

Discussion

These calculations have shown that it is possible to use the Tanford–Kirkwood theory to account for the experimentally observed titration curve of lysozyme on the basis of the locations of the titratable groups, assumed to be located on the molecule in solution in the positions given by the structure in the crystalline state. To do so, however, it has been necessary to treat one of the parameters in the theory as an empirical parameter, and it has also been necessary to exclude five of the titratable groups from calculation,[2] as they are clearly subject to strong influence from effects other than interaction between charged groups. These requirements, and additional questions to be raised subsequently in this discussion, detract from the apparent success of the theoretical calculation.

The most serious difficulty is probably associated with the depth parameter d, for the theory has no predictive value at all if this is an empirical parameter. If, for example, one wanted to predict the effect of a nearby charged group on the pK of a group in the active site of an enzyme one could obtain almost any value one chooses by adjusting the value of d. Moreover, small values of d would seem to be physically uninterpretable in terms of the Kirkwood model. Thus the value of $d = 0$ used by Orttung (1970) in the calculation for hemoglobin cannot be interpreted as indicating that the interacting charges are at the surface of the molecule. The treatment of charges as point charges and of the solvent as a continuum requires that d have a finite value when the charges are at the surface, and the calculations on small molecules described earlier indicate that $d = 1.0$ Å is the minimum possible value.

The most likely explanation is that the Kirkwood model is not appropriate for proteins because the ionic groups often extend into the surrounding solvent sufficiently far so that the medium between them is pure solvent and the effect of the large dielectric cavity several ångstroms away is minimal. The results with small molecules indicate that each charge must still be considered as being located in a cavity of low dielectric constant, at a depth of 1 Å below the surface, but the cavities are small and interacting charges would in most cases be located on *separate* cavities with solvent in between. No theoretical treatment for such a model has been reported.

The second difficulty is the unequivocal evidence presented for significant pK shifts as a result of interactions other than charge interactions. The most striking example is the pK of 6.3, nearly 2 pK units above the intrinsic pK, assigned to glutamic acid residue 35. This phenomenon could not have been predicted. The carboxyl group in question is accessible to water, and, while there are indeed many nonpolar residues in the vicinity, they do not envelop the group to a sufficient extent to make its environment uniquely abnormal. The existence of this major unexplained shift in pK and the smaller shifts we have found it necessary to ascribe to the α-amino group and the tyrosine residues raises the question of whether such unpredictable interactions may not affect most acidic and basic groups on the molecule. There is no way to answer the question. The fact that the effective pK of the lone histidine residue was predicted correctly on the basis of charge interactions alone, for example, does not constitute meaningful evidence because of the empirical value of d that was employed.

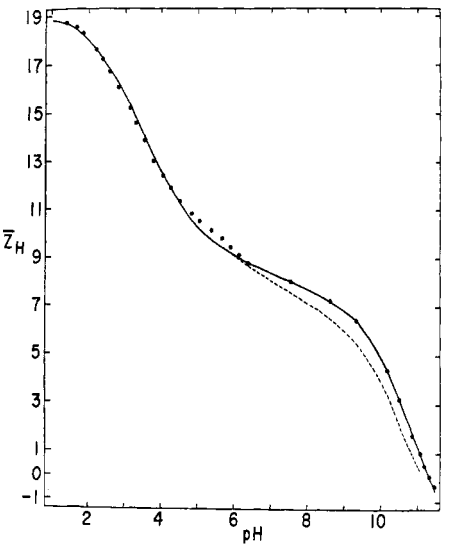

FIGURE 4: Calculated and experimental titration curves at ionic strength 1.0 m. The solid line is calculated as described in the text with five pK values assigned on the basis of other information. The dashed line is calculated entirely on the basis of the Tanford–Kirkwood theory, with the exception that glutamic acid residue 35 has been given a preassigned pK of 6.3. The points represent experimental data.

A problem of a different kind is raised by the calculation of a very low pK for the terminal α-COOH group. This agrees with the experimental observation that lysozyme possesses such a group. However, the work of Rupley *et al.* (1967) indicates that a very acidic group, having a pK around 1.8 in the native unbound protein, contributes to the pH dependence of substrate analog binding. The terminal α-COOH group, however, is far removed from the binding site. This does not in principle prevent identification of the α-COOH group with the substrate-influenced group because binding could induce a conformational change that would affect the group despite its distance from the binding site. However, the crystallographic studies of Blake *et al.* (1967) do not support this possibility. The possibility that there are two groups on lysozyme with a very low pK is remote, as previously discussed, and the possibility that the calculation of the pK of the α-COOH group is erroneous must therefore be considered seriously. One way this could have occurred is if the very close proximity of the α-COOH group and the lysyl amino group of residue 13, indicated by the crystal structure, does not apply to the lysozyme molecule in solution. This is actually quite a likely possibility: there is no compelling reason why the side chain of lysine-13 should not extend freely into the

[2] The groups were excluded in the sense that their pK values were not obtained on the basis of the calculation. Their charges were of course included in the calculation of the pK's of other groups.

solution when a large solvent volume is available to it. The phenomenon suggested here may again constitute a general impediment to the accurate prediction of pK's of individual groups: surface groups of a protein molecule may not be in the same positions in solution as in the crystalline state and even small shifts in position can significantly alter the magnitude of electrostatic interactions.

It may be noted in this connection that there is generally a significant effect of pH on the solubility of crystalline proteins. Application of the principle of linked functions (Wyman, 1964) then *requires* that the state of titration of the protein in the crystal be different from its state of titration in the solution at equilibrium with it, as may be seen by substituting the solubility constant for K_D in eq 1. This phenomenon has been discussed previously in some detail by Rupley (1968, 1969).

None of these difficulties should be interpreted as an argument against the general principle that interactions between charged groups have the greatest influence in determining the differences between the actual pK's of acidic and basic groups and their intrinsic pK values. Indeed, the effect of ionic strength can be interpreted on no other basis. It should also be noted, with reference to Table I, that the titration curve in the acid region is consistent with depressed pK values for most of the groups that titrate there, even if the assignment of actual values to particular groups may not be correct.

The results we have presented do show however that one cannot expect to be able to make an accurate prediction of the pK of a particular group, such as one might wish to make in the investigation of the active site of an enzyme. In particular, the results suggest that the Tanford–Kirkwood procedure, which has proved to be capable of giving accurate results for small molecules, has no clear advantage over simpler techniques when applied to proteins.

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