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Ionization Behavior of the Catalytic Carboxyls of Lysozyme. Effects of Ionic Strength†

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ABSTRACT: The pH difference titration of the β-ethyl ester derivative of the Asp-52 residue of lysozyme relative to native lysozyme has been obtained. The difference curve reflects the ionization behavior of both Asp-52 and Glu-35. Four microconstants describe the ionizations of the two interacting catalytic groups. The macroscopic ionization constant, including all forms of the enzyme, for Asp-52 is 4.5 while that for Glu-35 is 5.9 in 0.15 M KCl at 25°. The ionization of each group is strongly dependent on ionic strength in a manner opposite to that found for simple carboxylic acids. A Debye–Hückel treatment of the electrostatic potential from the rest of the enzyme adequately accounts for the ionic strength dependence. The results indicate that lysozyme in KCl solution has a net positive charge on the protein surface of about 6 to 7 over the pH range 4–7. If lysozyme were not charged and the two groups did not interact, Asp-52 and Glu-35 would have pK^0's of 5.3 and 5.8, respectively.

Recently we prepared, isolated, and identified a derivative of hen egg-white lysozyme which consists of the β-ethyl ester of the catalytic residue aspartic acid 52 (Parsons *et al.*, 1969; Parsons and Raftery, 1969). By subjecting this well-characterized derivative and native lysozyme to a differential measurement we could hope to gain detailed knowledge about the active site region in which effects from the rest of the protein were cancelled out. An obvious comparison to make when dealing with a singly esterified enzyme is a proton difference titration. It is worth noting that this is a preferred comparison even if another method such as pH-dependent perturbed protein ultraviolet (uv) absorbance is available. This is so because the hydrogen ion titration has a predictable stoichiometric span. Accordingly, more complex difference curves can be fitted with confidence.

Little information has been available about aspartic acid residue 52 (Asp-52). Kinetic data suggest that its pK is between 3.5 and 4.5 in the catalytic complex (Rupley, 1967; Parsons *et al.*, 1969; Rand-Meir *et al.*, 1969). A group with an apparent pK of about 3.2 affects the tryptophanyl chromophores (Donovan *et al.*, 1961; Lehrer and Fasman, 1967). A group of about pK 3.6 is the only acidic group to affect the electrophoretic mobility (Beychok and Warner, 1959), and two groups of pK 3.2 become normalized in guanidine hydrochloride solution (Donovan *et al.*, 1960). None of these effects, which may be due to several different groups, have been assigned to Asp-52. It will be apparent from the results presented in this paper that these effects do not arise from Asp-52. However, other unassigned observations which will be discussed below probably originate from this amino acid residue.

In contrast to Asp-52, information about the other catalytic residue, glutamic acid 35 (Glu-35), has been obtained previously. A single carboxyl of pK 6.0–6.5 appears in pH titrations (Sakakibara and Hamaguchi, 1968), becomes normalized in guanidine hydrochloride solution (Donovan *et al.*, 1960), perturbs tryptophanyl chromophores (Ogasahara and Hamaguchi, 1967; Donovan *et al.*, 1961; Lehrer and Fasman, 1967), affects the binding of inhibitors and substrates (Dahlquist *et al.*, 1966; Lehrer and Fasman, 1966; Rupley, 1967; Rand-Meir *et al.*, 1969), and affects the proton magnetic resonance chemical shift values of bound inhibitors (Dahlquist and Raftery, 1968). The X-ray crystallographic results (Blake *et al.*, 1967, 1965) place Glu-35 in a hydrophobic region of the enzyme consistent with all of these effects.

This paper reports on some aspects of the pH difference titration of the Asp-52 ester derivative. Four microconstants for the ionization of Asp-52 and Glu-35 in the native enzyme have been accurately determined in solutions containing 0.02–0.50 M KCl at 25°. A previous communication briefly described the results in 0.15 M KCl (Parsons and Raftery, 1970). Subsequent papers (Parsons and Raftery, 1972a,b) report on the effects of temperature and of inhibitors and substrates

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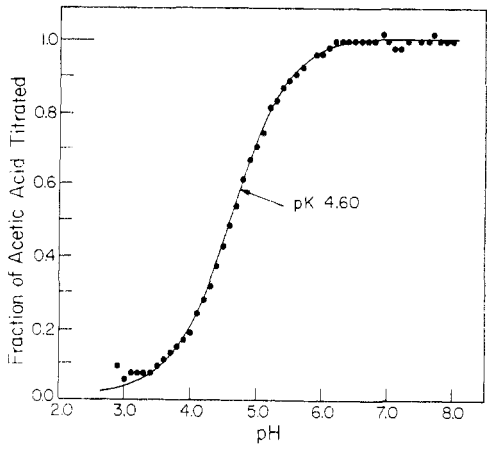

FIGURE 1: The difference titration of 2×10^{-4} M acetic acid in 3.5×10^{-4} M lysozyme, at 25° in 0.15 M KCl. The determined pK of 4.60 is the value expected under these conditions.

on the ionization properties of the catalytic carboxyl groups of the enzyme.

Experimental Section

Lysozyme was obtained from Sigma Chemical Co. (lot 28B-8120). Chromatographically pure native enzyme was prepared directly from the commercial lysozyme. The Asp-52 ester derivative was prepared from 10 g of lysozyme by reaction with triethyoxonium fluoroborate and isolated chromatographically as previously described (Parsons *et al.*, 1969). Both preparations were dialyzed separately for two days at 4° against many changes of 10^{-3} M KCl, passed through Dowex 1 (Cl^- form), and lyophilized. This preparation (1.1 g) of the derivative was 4% enzymatically active, compared to native enzyme, when assayed using *M. lysodeikticus* (Miles Laboratories) as substrate (Shugar, 1952); that is, its composition was 96% Asp-52 β-ethyl ester derivative and 4% native lysozyme.

Native or modified lysozyme (15 mg/ml) in carbonate-free 0.15 M KCl was filtered (Millipore Filter Corp., 0.45 μ pore size) to give a clear colorless solution at pH 6. Because of the increasing ionic strength throughout the titration, as explained below, solutions for the titration in about 0.02 M KCl were prepared in 0.01 M KCl. The uv difference spectrum (320–260 nm) of native *vs.* modified lysozyme was obtained on a Cary Model 14 spectrophotometer utilizing a 0.1-OD slidewire and matched uv cells of 0.5-mm path length (Research and Industrial Instruments Division of Beckman, Fullerton, Calif., Model UV 10). The two protein concentrations were adjusted until the deviation from the blank (lysozyme *vs.* lysozyme) was small. The actual absorbance at 280 nm was 1.9 so that a deviation of 0.004 absorbance at 280 nm in the difference spectrum represented a mismatch in protein concentrations of only 0.2%.

The pH titration vessel consisted of a 13-mm inner diameter test tube, bulged at about 25 mm from the bottom, which was fitted with 9-mm outer diameter combined calomel–glass electrodes (Radiometer, GK 2302C), a magnetic stirring bar, a nitrogen inlet made from polyethylene tubing, and a polyethylene U-bent titrant delivery tube with a double crook in it above the sample level. The bulge and the crook were necessary to prevent the protein solution from rising up the sides of the vessel or electrode by capillary action. The titration vessel was thermostated to within ±0.02°. Nitrogen was saturated with water vapor at the same temperature and the line leading to the titration vessel thermostated. The pH meter was a Radiometer Model 26. Standard buffers (pH 4 and 7) were obtained from Beckman. In standardizations and titrations ample time was allowed for thermal equilibration of the glass electrode and for removal of CO_2. The response of the electrodes was checked after each titration to ensure that it was not impaired by the protein.

A 2.00-ml portion of either the native or modified lysozyme was pipetted carefully into the vessel using the same clean dry pipette. For titrations at salt concentrations above 0.15 M the appropriate weight of crystalline KCl was added. The solution was equilibrated 1 hr after which the pH was raised to 9.1 with about 0.04 ml of 0.150 N KOH (Baker and Adamson, reagent special, "low carbonate"). The protein solution was then titrated down in pH with 0.150 N HCl utilizing an "Agla" micrometer syringe of 0.5000-ml capacity (Burroughs Wellcome and Co., London). All joints in the titrant delivery system were sealed with wax, and a drop of glycerine was applied to the syringe plunger at the barrel to prevent loss of titrant by capillary action. The volume of HCl required to reach a particular 0.100 pH unit on expanded scale was recorded after allowing time for mixing and temperature equilibration. The micrometer reading at a 0.100 pH unit for the lysozyme titration was subtracted from the corresponding reading for the Asp-52 ester derivative to give a difference titration curve.

The effect of ionic strength on the Asp-52 ester difference titration was observed at 0.02, 0.15, 0.22, 0.30, and 0.50 M KCl. Because 0.150 N titrant was used for all these titrations, the stated KCl concentrations varied by about ±0.007 M at μ values other than 0.15 M. The value of 0.02 M KCl is the approximate ionic strength at pH 4 which was present in the solutions originally prepared in 0.01 M KCl. The enzymatic activity of the Asp-52 ester preparation was checked after the titration. Also, a control titration of lysozyme *vs.* lysozyme plus potassium acetate was performed.

Results

Figure 1 shows that less than one mole-ratio equivalent of acetate easily could be determined accurately in the presence of a "background" of the 11 protons in lysozyme titrating below pH 7. The points are the experimental difference titration while the line is an ideal titration curve. The determined pK of 4.60 is that expected in 0.15 M KCl.

In matching the native lysozyme and Asp-52 ester protein concentrations, the molar extinction coefficients of both would best be identical. However, since aspartic acid residue 52 is close to several tryptophane residues, the uv difference spectrum at pH 6 of closely matched samples exhibited a typical lysozyme tryptophanyl difference spectrum (Donovan *et al.*, 1961; Dahlquist *et al.*, 1966) with a peak at 293 nm, a trough at 290 nm, and no peak at 280 nm. The difference was relatively small though, and identical absorbancies at 280 nm and about pH 6 meant essentially identical protein concentrations for native lysozyme and the derivative.

A typical titration in 0.15 M KCl from pH 9.000 to pH 3.000 resulted in acid uptake totalling 0.1094 ml for native lysozyme and 0.0979 ml for the derivative. The difference in water volumes between the two solutions toward the end of the titration required a very small correction. After the titration the enzymatic activity of the Asp-52 ester preparation remained 4% that of native lysozyme, indicating no ester hydrolysis during the titration. Identical results were obtained

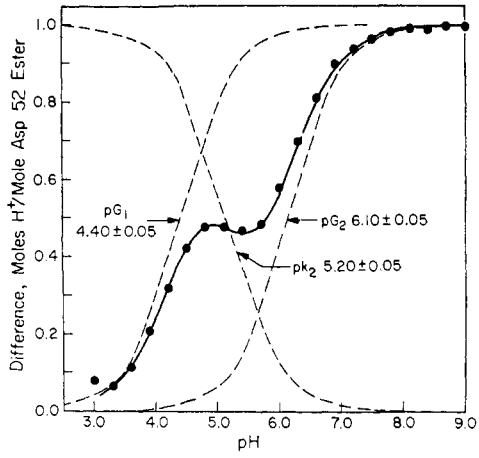

FIGURE 2: Difference titration of 2.00 ml of lysozyme *vs.* the Asp-52 ester derivative, both 15 mg/ml (about 1×10^{-3} M), in 0.150 M KCl at 25.0°. The total uptake of 0.150 N HCl from pH 9.000 to pH 3.000 was 0.1094 ml for lysozyme and 0.0979 ml for the derivative. The expected difference due to one amino acid side chain ester is 0.0126 ml which has been normalized to a span of one. The data points were fitted with the sum of the two positive and one negative ideal titration curves shown as dashed lines. The values of pG_1 and pG_2 are close to the ionization constants of Asp-52 and Glu-35, respectively, and pk_2 is the value for Glu-35′ in the derivative.

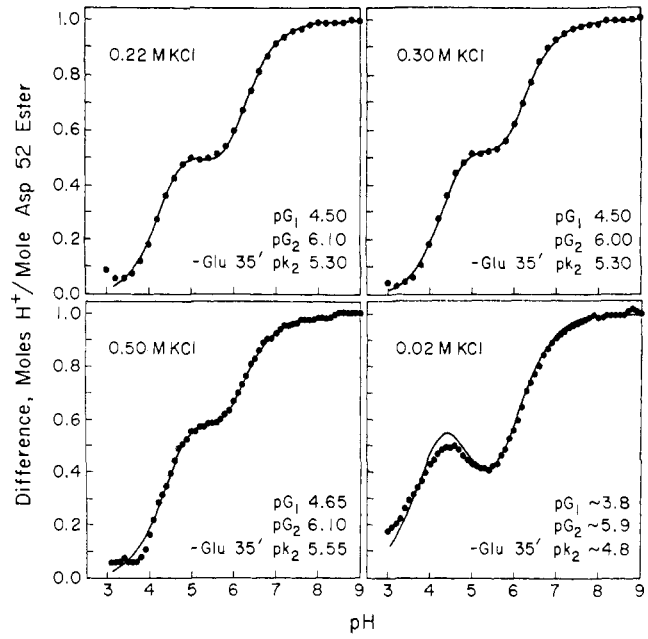

FIGURE 3: The difference titrations in approximately 0.22, 0.30, 0.50, and 0.02 M KCl at 25°. The two bottom curves were obtained from the same stock solutions originally made up in 0.01 M KCl. A separate difference titration in 0.50 M KCl gave titration constants of 4.70, 6.00, and 5.40. The data for 0.02 M KCl cannot be fitted as well with the three titration constants because of the possible increased importance of generalized electrostatic interactions and a significantly changing ionic strength.

whether the titration was carried out from high to low pH or *vice versa*. Also, titrations at 15 and 5 mg per ml of protein yielded the same difference curve. All the titrations reported here were performed from high to low pH at 15 mg/ml of protein.

The difference points in Figure 2 show that titration of the "excess" Asp-52 side chain is more complicated than the simple curve shown in Figure 1. This is readily explained as resulting from the perturbation of a second group (besides Asp-52) in the ester derivative. That is, there apparently are three ionizations reflected in the difference data. Their resolved contributions are shown by the dashed lines in Figure 2.

The perturbed group is certainly Glu-35 since it is located quite close to Asp-52 in the tertiary structure. Absence of the negative electric field from Asp-52 at pH 5–6 in the ester derivative would lower the pK of Glu-35. His-15 with a pK of 5.8 is probably the only other group in lysozyme with a pK in this range (Bradbury and Wilairat, 1967), but it is on the other side of the molecule. Thus in the first rising segment of the difference data between pH 3.0 and 4.8 the titration of Asp-52 predominates. In the plateau region Glu-35′ in the derivative titrates and partially cancels the Asp-52 titration. The proton difference then continues to rise toward one as the ionization of Glu-35 in the native enzyme predominates between pH 5.7 and 9.0. Other interpretations of Figure 2 are possible but extremely unlikely.

The theoretical volume of acid uptake due to the excess Asp-52 side chain can be calculated from the molarity of the enzyme solution obtained from its 280-nm absorbancy (Sophianopoulos *et al.*, 1962), the per cent inactivity of the Asp-52 ester preparation, and the normality of the titrant. In Figure 2, one mole-ratio of the derivative corresponds to 0.0126 ± 0.0002 ml. Utilizing this theoretical span to the difference titration, the points were fitted with the sum of the two positive and one negative ideal titration curves shown in Figure 2. As will be discussed below, the positive curves for the interacting Asp-52 and Glu-35 system in the native enzyme determined "titration" constants G_1 and G_2, respectively. The pG values are nearly, but not quite, correct values for individual Asp-52 and Glu-35 ionizations. The negative curve for Glu-35′ in the ester determines a true dissociation constant k_2.

The values for G_1, G_2, and k_2 were guessed until superposition of the summed curve on the experimental points gave a close fit as visually determined. Figure 2 was best fitted by pG_1 equal to 4.40, pG_2 equal to 6.10 in native lysozyme, and pk_2 equal to 5.20 in the derivative. A repeat titration under the same conditions on a separate preparation of the protein solutions yielded a curve which was fitted as well as Figure 2 with values of 4.35, 6.05, and 5.25, respectively.

The data from titrations in KCl concentrations varying from 0.02 M to 0.50 M are shown in Figure 3. The data shown for 0.02 M and 0.50 M KCl were obtained from identical solutions with crystalline KCl added to the latter titration sample in order to attain about 0.50 M KCl. A repeat titration on a separate preparation in 0.15 M KCl made up to 0.50 M KCl gave titration constants of 4.70, 6.00, and 5.40, in good agreement with the curve shown. All difference results were fitted as described above.

Discussion

Interpretation and Analysis of the Difference pH Titration Curve. Usually, ideal titration curves are not expected in proteins because of a variable electrostatic potential on the molecule. A general scheme would require multiplication of each individual ionization constant by some complicated function of the charge interactions (Tanford, 1961). Although progress has been made (Orttung, 1970; Tanford and Kirkwood, 1957) exact calculation of the local electrostatic potential in a protein is presently most difficult and somewhat un-

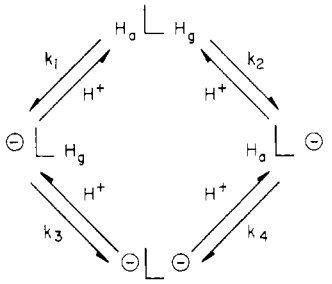

FIGURE 4: The two catalytic carboxyls of lysozyme considered as a dibasic acid. Subscripts a and g refer to Asp-52 and Glu-35. The predominate mode of ionization in the enzyme follows microconstants k_1 and k_3 with $^{\ominus}LH_g$ the catalytically active species.

certain. In any case, as discussed next, it is fortunate that in lysozyme we usually can ignore the change of this potential with pH to a first approximation.

Beychok and Warner (1959) showed that lysozyme binds anions at 0.15 M ionic strength such that its electrokinetic potential changes little below pH 6. Also, Carr (1953) measured the extent of chloride ion binding in about 0.012–0.019 M and 0.10–0.12 M ionic strength solutions. He found that about the same number of chloride ions was bound at both ionic strengths at pH 3. Thus, on the average, nearly one chloride ion binds for each proton bound below pH 6 over a wide range of ionic strength. Furthermore, only two protons titrate between pH 6 and 9 and one of them is Glu-35 itself (Sakakibara and Hamaguchi, 1968). Therefore, the total charge on lysozyme changes very little below pH 9. One could hope that the relatively weak interactions with distal lysozyme-bound charged species would average to give a nearly constant electrostatic potential in the region of the active site in solutions of sufficient ionic strength.

Esterification of one carboxyl generally will affect the ionization of distal titratable groups only slightly, and the titrations of distal groups will cancel closely in a difference pH titration. If we assume that one group interacts strongly with the esterified carboxyl and is the only other significantly perturbed group, and also that the conditions of the preceding paragraph hold, we can write eq 1 for the difference result.

$$\Delta\bar{h} = \frac{G_1}{H^+ + G_1} + \frac{G_2}{H^+ + G_2} - \frac{k_2}{H^+ + k_2} \qquad (1)$$

Here G_1 and G_2 are the "titration" constants for the two strongly interacting groups in the native protein calculated as if they were an equivalent mixture of two simple monovalent acids, k_2 is the new dissociation constant for the group perturbed by the esterification, and $\Delta\bar{h}$ is the differential proton uptake. These constants are not in general based on "intrinsic" constants but may have been raised or lowered by electrostatic, hydrogen bonding, or hydrophobic effects.

The very close fit of the data to eq 1 in solutions containing at least 0.15 M KCl under the restriction of an independently determined difference span lends strong support to the adequacy of this scheme. The curve obtained in approximately 0.02 M KCl is also in fact good confirmation of the analysis. In the low ionic strength solution where the pH-variable potential is possibly more important (and also where the salt concentration was changing significantly throughout the titration) the data can still be approximately fitted with only three titration constants. Even in a low-salt solution no other specific group titratable between pH 3 and 9 interacts strongly with Asp-52 or Glu-35.

TABLE I: Ionic Strength Dependence of the Microconstants for the Asp-52 and Glu-35 Ionizations.[a]

Ionic strength, M	0.02	0.15	0.22	0.30	0.50
pk_1 (Asp-52)	3.8	4.40	4.56	4.56	4.69
		4.46			4.77
pk_2 (Glu-35′)	4.8	5.25	5.30	5.30	5.55
		5.20			5.40
pk_3 (Glu-35)	5.9	6.00	6.04	5.94	6.06
		6.03			5.93
pk_4 (Asp-52)	4.9	5.15	5.30	5.20	5.20
		5.29			5.30

[a] At 25° in KCl as calculated by eq 2 and 3 applied to Figures 2 and 3, as well as two figures not shown, by assuming k_2.

The two "titration" constants, G_1 and G_2, for a dibasic acid are related to the two molecular dissociation constants, K_1 and K_2, describing the first and second stages in the titration, by eq 2a and 2b. The two molecular dissociation constants

$$K_1 = G_1 + G_2 \qquad (2a)$$

$$K_1K_2 = G_1G_2 \qquad (2b)$$

are dependent upon four microconstants for the individual groups according to eq 3a and 3b. A complete discussion of

$$k_1 + k_2 = K_1 \qquad (3a)$$

$$\frac{1}{k_3} + \frac{1}{k_4} = \frac{1}{K_2} \qquad (3b)$$

these relationships can be found in Edsall and Wyman (1958a) and in Simms (1926).

The two ionization pathways with their four microconstants are illustrated in Figure 4 for the interacting Asp-52–Glu-35 system. H_a represents a proton bound to the Asp-52 side chain and H_g a proton bound to the Glu-35 side chain. Because the Asp-52 proton is predominately the first to ionize we can assume that constants k_1 and k_3 dominate both the molecular dissociation constants, K_1 and K_2, and the titration constants, G_1 and G_2, respectively.

Provided that we know one of them, all four microconstants can be calculated from the titration constants by utilizing eqs 2 and 3. We already have obtained one of the four if we may identify the titration constant for Glu-35′ in the Asp-52 ester derivative with k_2 of Figure 4. This seems to be a reasonable assumption since inspection of the lysozyme model reveals that there is room to accommodate the ethyl group of the ester without serious interaction with Glu-35. If there is an effect from the ethyl group it should be in providing a more hydrophobic environment for Glu-35′, thus raising its pk_2 more toward the value in the presence of ionized Asp-52. Thus this assumption leads to a minimum estimate of the electrostatic interaction between Asp-52 and Glu-35.

The calculations have been carried out on this basis and are listed in Table I. It should be noted that the Asp-52 and

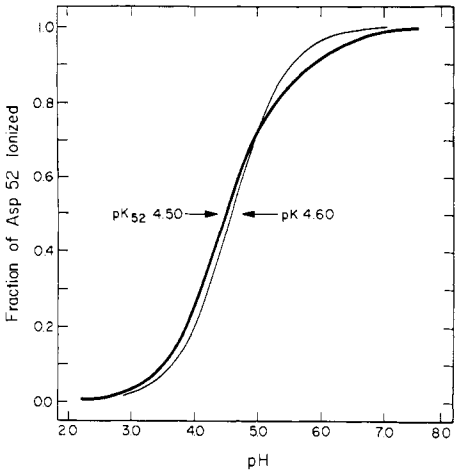

FIGURE 5: The macroscopic ionization of Asp-52 in native lysozyme. The heavy curve was calculated from eq 4 using the average of the microconstants determined at 25° in 0.15 M KCl. One-half of the residue is ionized at pH 4.5. The lighter curve is an ideal titration curve that might be considered as the best fit to a direct observation of the Asp-52 ionization. Its pK is 4.6. The macroscopic Glu-35 ionization curve would be of similar shape with the point of half-ionization occurring at pH 5.9.

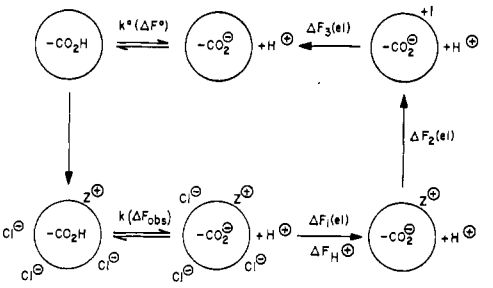

FIGURE 6: A Born–Haber cycle relating an observed microconstant k_1 or k_2 to a hypothetical ionization k^0 in uncharged lysozyme at zero ionic strength. $Z^{\oplus}$ is the net charge on the surface of the protein and each ΔF is defined in the text.

Glu-35 ionizations overlap only moderately since pk_1 and pk_3 differ from pG_1 and pG_2, respectively, by less than 0.10 pH unit in all cases. Therefore, the titration behavior of Asp-52 and Glu-35 determined from Figures 2 and 3 can be in error only slightly if the value assumed for k_2 is somewhat in error. Allowing a ±2% error in determining the theoretical titration span, a ±2% error in the experimental difference data, the slightly nonideal shape expected of the difference titration curve, and the possible error in k_2, we estimate a reasonable error in the absolute values of pk_1 and pk_3 to be ±0.1 pH unit. The absolute values of pk_2 and pk_4 are more uncertain. Relative values for pk_1, pk_2, and pk_3 are considerably more certain. The averaged microconstants corresponding to Figure 4 for Asp-52 and Glu-35 at 25° in 0.15 M KCl are pk_1 = 4.43; pk_2 = 5.22; pk_3 = 6.01; and pk_4 = 5.22.

Shape of the Asp-52 Ionization Curve. We can calculate the macroscopic shape of the Asp-52 ionization curve by utilizing the microconstants and eq 4, which is derived from mass

$$\text{fraction Asp-52 ionized} = \frac{k_1[(H^+) + k_3]}{k_1[(H^+) + k_3] + (H^+)[(H^+) + k_2]} \quad (4)$$

balance. As shown by the heavy line in Figure 5 the titration is more shallow than an ideal one, and it is also asymmetric. The point of half-ionization of Asp-52, which includes all lysozyme species both with and without Glu-35 protonated, occurs at pH 4.5 at 25° in 0.15 M KCl. If the actual curve were approximated by an ideal one such as the lighter line in Figure 5, a pK of 4.6 would be estimated. A similarly shaped curve is obtained for Glu-35 from an equation analogous to eq 4.

Ionic Strength Effects on the Microconstants. Table I demonstrates that an increase in the ionic strength of the solution increases pk_1 and pk_2 considerably. It should be noted that there is no evidence for a significant salt-dependent conformational change in lysozyme (Davies *et al.*, 1969; Imoto *et al.*, 1969; Praissman and Rupley, 1968). These changes in pk's are in the direction opposite to that which occurs for simple carboxylic acids in increasing ionic strength solutions (Donovan *et al.*, 1959). The trend indicates a positive electrostatic potential from the rest of the protein.

It is of some interest to us to be able to quantitate these effects because they derive from the electrostatic nature of the lysozyme active site. The three-dimensional structure of lysozyme indicates that all the other ionizable groups are more or less distributed evenly on the surface of the molecule. Also, since they are buried within the enzyme, Asp-52 and Glu-35 cannot be approached closely by ions in solution. There will be little localization of the ion atmosphere around their side chains. If we assume that electrostatic effects on the active site from the rest of the enzyme can be formalized as a generalized potential we can use the Debye–Hückel treatment to estimate the consequences. Such a model is appropriate only for the two microscopic ionizations which occur when the other catalytic group is protonated.

It should be noted that in the more complete treatment of protein charge interactions given by Tanford and Kirkwood (1957), deviations from the Debye–Hückel treatment for particular ionizable groups occur to an extent dependent on the distance of the residue from the center of the protein. The ionic strength dependent electrostatic potential acting on a particular carboxyl group *precisely at the center* of a spherical protein would be accurately described by the Debye–Hückel model, even for a grossly nonsymmetrical distribution of surface charge. Because the crystallographic structure of lysozyme indicates that Asp-52 is near the center and that Glu-35 is about halfway to the surface of the molecule taken as a sphere, we expect that deviations will be small.

In considering the effects of charge interactions we will relate the observed ionization to a hypothetical ionization occurring in *uncharged lysozyme* at zero ionic strength by means of the Born–Haber cycle shown in Figure 6. Here step k is an observed ionization in the presence of KCl and corresponds to microconstant k_1 or k_2, Z is the net number of (positive) charges on the protein *surface*, ΔF_n(el) corresponds to the change in electrostatic free energy of the ionized Asp-52 or Glu-35 lysozyme ion for each respective thermodynamic step, ΔF_H corresponds to the change in hydrogen ion activity from its standard state at zero ionic strength, and k^0 is the value which k would exhibit in uncharged lysozyme at zero ionic strength. It should be noticed that there is no electrostatic free energy term shown for the left vertical step in Figure 6 since the dipolar energy will be comparatively small. Thus ΔF^0 corresponding to step k^0 is related to ΔF_{obsd} for step k by eq 5.

$$\Delta F^0 = \Delta F_{obsd} + \sum_i \Delta F_n(\text{el}) - \Delta F_H \quad (5)$$

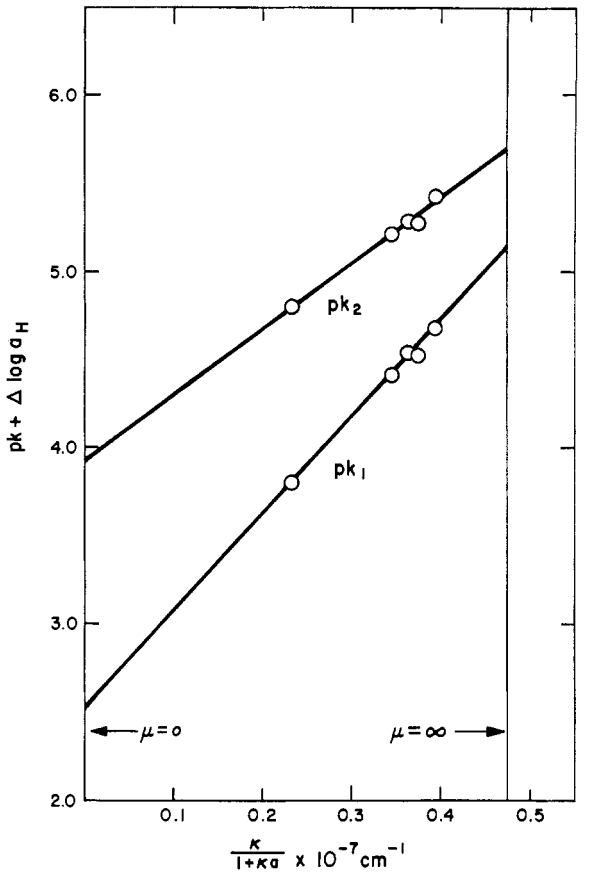

FIGURE 7: The Debye–Hückel theory with an empirical hydrogen ion activity correction applied to the effect of the lysozyme charge on the ionizations of Asp-52 and Glu-35 (eq 11), each ionizing when the other is protonated, at 25° in varying concentrations of KCl. The value of a has been taken as 21 Å. The slopes indicate that Asp-52 sees +7 charges and Glu-35 sees +6. The values which pk_1 and pk_2 would have at zero ionic strength if the rest of the lysozyme molecule were not charged are pk_1^0 equal to 5.3 and pk_2^0 equal to 5.8.

The Debye–Hückel approximation (Edsall and Wyman, 1958b) immediately allows us to write eq 6, 7, and 8 for each respective ΔF_n(el) per single lysozyme ion going counterclockwise around Figure 6.

$$\Delta F_1(\text{el}) = \frac{(z-1)^2\epsilon^2}{2D_w}\left(\frac{\kappa}{1+\kappa a}\right) \quad (6)$$

$$\Delta F_2(\text{el}) = \frac{(z-1)^2\epsilon^2}{2D_w}\frac{1}{b} \quad (7)$$

$$\Delta F_3(\text{el}) = \frac{\epsilon^2}{2D_w}\frac{1}{b} \quad (8)$$

Here ϵ is the protonic charge, D_w is the dielectric constant of the surrounding aqueous medium, b is the radius of the protein taken as a conducting sphere, a is the closest approach which a small spherical ion of radius r_s can make to the center of the protein ($a = b + r_s$), and κ is the reciprocal distance of the ionic atmosphere and is a function of the ionic strength. It is given by

$$\kappa = \left(\frac{4\pi\epsilon^2}{D_w\mathbf{k}T} \times \frac{2N}{1000}\right)^{1/2}(\mu)^{1/2} \quad (9)$$

TABLE II: Debye–Hückel and Acidity Parameters.

Ionic Strength, M	$\kappa \times 10^{-7}$,[a] cm^{-1}	$\frac{\kappa}{1+\kappa a} \times 10^{-7}$,[b] cm^{-1}	Δ log a_H[c]
0.00	0.000	0.000	0.000
0.02	0.463	0.234	−0.002
0.15	1.272	0.346	−0.015
0.22	1.540	0.364	−0.022
0.30	1.800	0.376	−0.030
0.50	2.32	0.395	−0.050
∞	∞	0.476	

[a] At 25°. [b] a equal to 21 Å. [c] Taken from Schwabe (1967), equivalent to ΔpH.

Here **k** is the Boltzman constant, T is the absolute temperature, N is Avogadro's number, and μ is the ionic strength. Equation 6 calculates the increase in electrostatic free energy due to the loss of the surrounding ion atmosphere. Equations 7 and 8 calculate the energy involved in decharging the surface of the lysozyme ion as a result of the change in the self-potential of ions with charge $z - 1$, 0, and −1.

In describing ΔF_H we prefer an empirical approach. Schwabe (1967) has measured the changes in hydrogen ion activity as a function of added KCl and these are listed in Table II as Δ log a_H. It can be seen that the largest correction is only 5 or 7% of the observed change in pk_1 or pk_2, respectively (Table I). ΔF_H is given by eq. 10

$$\Delta F_H = 2.3RT\Delta \log a_H \quad (10)$$

After substituting eq 6, 7, 8, and 10 into eq 5, then rearranging and cancelling terms and converting free energy to pk we obtain

$$pk + \Delta \log a_H = pk^0 + \frac{(Z-1)^2\epsilon^2}{4.6\mathbf{k}TD_w}\left(\frac{\kappa}{1+\kappa a}\right) - \frac{(Z^2-2Z)\epsilon^2}{4.6\mathbf{k}TD_w}\frac{1}{b} \quad (11)$$

Each of the symbols has been defined above.

A plot of $pk + \Delta \log a_H$ *vs.* the ionic strength function $\kappa/(1+\kappa a)$ should yield a straight line from which we can obtain Z and pk^0. To determine each $\kappa/(1+\kappa a)$ as given in Table II we have taken b equal to 19 Å, a number evaluated from the diffusion constant of lysozyme (Warner, 1954) and in harmony with the crystallographic dimensions (Blake *et al.*, 1965), and a equal to 21 Å, a number based on the usual 2 Å radius for the aqueous chloride ion (Edsall and Wyman, 1958c). Figure 7 shows that a straight line fits the points well.

The least-squares slopes in Figure 7 are 5.41×10^{-7} cm for pk_1 and 3.68×10^{-7} cm for pk_2. From the slopes we can calculate that Z equals 6.9 for Asp-52 and 5.9 for Glu-35. Extrapolation to zero ionic strength predicts that pk_1 and pk_2 would have values of 2.5 and 3.9, respectively. At infinite ionic strength, their values would be 5.1 and 5.7, respectively. From the intercepts at either zero or infinite ionic strength we calculate that pk_1^0 is 5.3 and pk_2^0 is 5.8.

We should point out that the values of Z and pk^0 which are determined by eq 11 are only moderately sensitive to the radii chosen for a and b. With a equal to 23 Å and b equal to 21 Å we would estimate that Z equals 7.3 for Asp-52 and 6.2 for Glu-35. The extrapolated values for pk^0 are changed even less and yield the same numbers (to the nearest tenth) as before.

Thus, Asp-52 sees about 7 net positive charges and Glu-35 about 6, from the rest of the lysozyme molecule. Furthermore, if it were not for the large positive charge on lysozyme and for their mutual charge interaction, Asp-52 and Glu-35 would have pk^0's of about 5.3 and 5.8, respectively. We consider these numbers to be indicative of the "solvent" environments of Asp-52 and Glu-35. It is apparent that both carboxyl groups are in somewhat hydrophobic regions of the enzyme.

Conclusion

The two catalytic carboxyls of lysoyzme clearly can be regarded in their charge interactions as analogous to a strongly interacting dibasic acid. They are not perturbed significantly by any other particular group in the enzyme titrating between pH 3 and 9, but they are subject to a relatively constant, generalized electrostatic potential, which depends upon ionic strength, from the rest of the enzyme. Although the ionization constant of Asp-52 would appear normal on the basis of its observed pK, for example, from Figure 2 or 5, it is, in fact, not normal. Figure 7 demonstrates that Asp-52 is in a slightly hydrophobic environment which gives it an "intrinsic" pk^0 only somewhat lower than that of Glu-35. It should be remembered that if k_2 is in error it is in the direction making Glu-35 appear to be in a more hydrophobic environment than it actually is. These "solvent" determined pk^0's are lowered by the positive charge on lysozyme, with the Asp-52 pk_1 being lowered the most to 4.43 and the Glu-35 pk_2 being lowered to 5.22 in 0.15 M KCl. The negative charge on ionized Asp-52 then raises the ionization constant pk_3 of Glu-35 to 6.01. The final result is that an "intrinsic" ionization advantage of about 0.5 pH unit on the part of Asp-52 has been increased to 1.4 pH units (pK = 4.5–5.9, Figure 5) by electrostatic interactions.

The validity and quantitative correctness of the analysis undertaken here is supported by several pieces of evidence. The ionization constant determined for Glu-35 is in good agreement with previously determined values. As for Asp-52, using nuclear magnetic resonance techniques, Dahlquist and Raftery (1968) have observed a carboxyl in the lysozyme cleft with an apparent pK of 4.7 in both the free and inhibitor complexed enzyme Kowalski and Schimmel (1969) observed that the binding of freshly dissolved α-N-acetylglucosamine is affected by a group of about pK = 4.5 in the free enzyme. It is very likely that both effects arise from a carboxyl close to the primary binding subsite and that this group is Asp-52. The pK's determined by the other workers (but not assigned) are in good agreement with the value 4.6 in Figure 5 which would result from an observation which was directly dependent on the macroscopic ionization of Asp-52 as determined here. Furthermore, none of the above studies, some of which are relatively precise, have indicated a large deviation from ideality in the titration of either Asp-52 or Glu-35, thus supporting the adequacy of eq 1. Finally, the total charges on the lysozyme molecule over the pH range 3–7 determined from the slopes of Figure 7 are in good agreement with the values of about 6–7 determined by Beychok and Warner (1959) from electrophoretic mobilities.

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