ΔH even more positive in the presence of ionized Asp-52 (2.9 kcal/mole, Table II). The somewhat abnormally negative value for ΔS^0 in uncharged lysozyme of -22 e.u., however, is surprising. Even the uncorrected normal value for ΔS_{obsd} of -18 e.u. indicates a larger decrease in entropy than one might have expected for formation of an anion in a poorly solvating medium. This indicates that either water in the lysozyme cleft, or part of the protein structure, or both, is specifically oriented by the ionization of Glu-35.

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Ionization Behavior of the Cleft Carboxyls in Lysozyme–Substrate Complexes†

S. M. Parsons‡ and M. A. Raftery*

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 at 25° and 0.15 M KCl in the presence of several inhibitors and the substrate glycol chitin. The data reflect the ionizations of Asp-52, Glu-35, and Asp-101. The Asp-52 ionization changes very little in all the complexes while the Glu-35 ionization exhibits a pK of about 6.5 in the inhibitor complexes and a pK of 8.0 to 8.5 in the glycol chitin complex. Asp-101 in the native enzyme exhibits the expected pK changes due to hydrogen bond formation to bound inhibitors. Asp-101′ in the ester derivative complexes fails to form these hydrogen bonds.

The successful measurement of some properties of Asp-52 and Glu-35 in the free enzyme as reported in the two preceding papers (Parsons and Raftery, 1972a,b) prompted us to attempt similar determinations for complexes of lysozyme with inhibitors and a substrate. Such information clearly would be of utility in describing the roles of Asp-52 and Glu-35 in initiating catalysis.

The apparent pK of Glu-35 in NAG-βCH$_3$[1] and NAG$_3$ complexes with lysozyme has been determined to be about 6.5 by several methods (Dahlquist and Raftery, 1968; Dahlquist *et al.*, 1966). These complexes do not interact directly with the catalytic site. No physical information is available in the literature about the pK of Glu-35 when the enzyme is bound to substrates which interact intimately with the active site. As for Asp-52 there has been no pK previously assigned to it. The only data come from kinetic studies, which suggest a pK around 4. Since the binding of small inhibitors appears not to perturb the pK of Asp-52 (Dahlquist and Raftery, 1968; Dahlquist *et al.*, 1966) we expect it to exhibit a titration constant of about 4.4 in these complexes.

This paper reports on difference titrations carried out in the presence of methyl β-NAG, methyl β-chitobioside, chitotriose, methyl β(1–4)-chitotriosyl-β-D-glucopyranoside, and glycol chitin. The curves reflect the behavior of both

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[1] Abbreviations used are: NAG-βCH$_3$, methyl 2-acetamido-2-deoxy-β-D-glucopyranoside; NAG$_2$-βCH$_3$, methyl β-chitobioside; NAG$_3$, chitotriose; NAG$_3$-Glu-βCH$_3$, methyl β(1–4)-chitotriosyl-β-D-glucopyranoside; NAG-Glu-βC$_6$H$_4$NO$_2$, nitrophenyl 2-acetamido-2-deoxy-D-glucopyranosyl-β(1–4)-D-glucopyranoside.

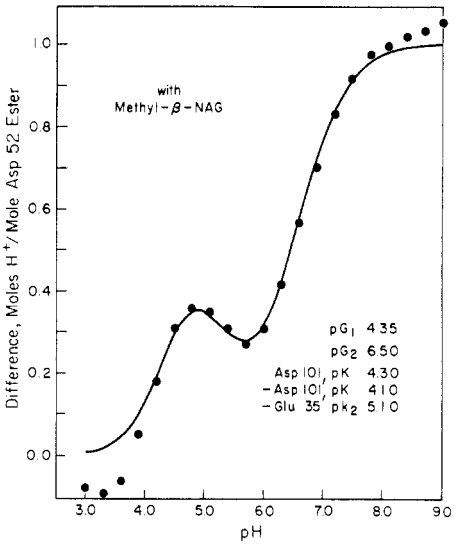

FIGURE 1: The difference pH titration in the presence of 122 mg of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside at 25° in 0.15 M KCl. The data required five titration constants for a close fit. The numbers pG_1, pG_2, and pk_2 have the same meaning as in Parsons and Raftery, 1972a. The pK value for Asp-101′ is for the ester derivative. Binding subsite C is filled.

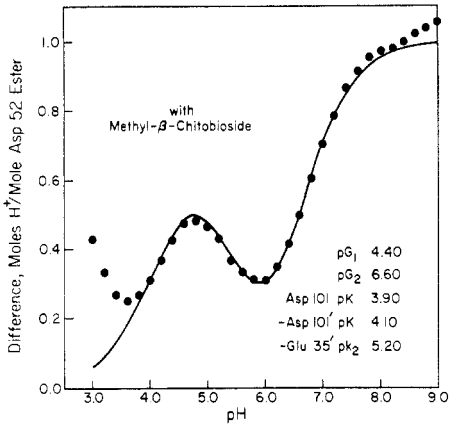

FIGURE 2: The difference pH titration in the presence of 20 mg of methyl β-chitobioside, at 25° in 0.15 M KCl. The data require five titration constants for a close fit. The numbers pG_1, pG_2, and pk_2 have the same meaning as in the preceding papers. The pK value for Asp-101′ is for the ester derivative. Binding subsites BC are filled.

catalytic carboxyls as well as Asp-101 under conditions where various binding subsites are occupied.

Experimental Section

Materials. Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (mp 202–204°) and methyl β-chitobioside (mp 287–288°) were gifts of F. W. Dahlquist. Glycol chitin was prepared by the method of Senzyu and Okimasu (1950). The product was vacuum filtered through medium grade sintered glass. Chitotriose and chitotetraose were prepared by partial acid hydrolysis (Rupley, 1964) of chitin (Sigma Chemical Co.) by gel filtration on Bio-Gel P-2 (Raftery *et al.*, 1969). All the above products were treated briefly with amberlite mixed bed ion-exchange resin (MB-1) in order to remove any titratable ions before the final lyophilization.

Methyl β(1–4)-chitotriosyl-β-D-glucopyranoside was synthesized using the transferase activity of lysozyme. Chitotetraose (5 g) was incubated 4 hr with 50 g of methyl β-glucoside (Sigma) and 0.5 g of lysozyme in 1 l. of 0.1 M acetate, pH 5.5 at 37°. The solution was acidified to pH 3, ultrafiltered to remove the enzyme (UM-1 membrane, Amicon Corp.), and stirred 1 day with 300 ml of MB-1 resin to remove the buffer and most reducing sugars. This solution was concentrated *in vacuo* and then gel filtered on a 10 × 180 cm column of Bio-Gel P-2 (200–400 mesh) in water. Fractions (100 ml) were collected and the chitosyl glucosides were located by their absorbance at 220 nm. The fractions corresponding to methyl β(1–4)-chitotriosyl-β-D-glucopyranoside were pooled and lyophilized. To remove the last traces of reducing sugars the product was taken up in water and passed slowly through a small column of MB-1. The glycoside was collected and lyophilized to yield 100 mg of product. The structure proof for similar transferase products has been described previously (Rand-Meir *et al.*, 1969).

The same preparations of lysozyme and of the Asp-52 ester that were used for the titrations in the two preceding papers were used here.

Methods. The preparation of matched protein solutions and the titration procedure have been described (Parsons and Raftery, 1972a). Weighed amounts of the solid sugars, except for glycol chitin, were added to the titration vessel before addition of the protein solutions. For methyl β-NAG, this was 122 mg; for methyl β-chitobioside, 20 mg; for chitotriose, 2.5 mg; and for methyl β(1–4)-chitotriosyl β-D-glucopyranoside, 10 mg. For the glycol chitin, 1 ml of a stock solution (15 mg/ml) in 0.15 M KCl was pipetted carefully into the titration vessel.

Difference pH titrations were carried out at 25.0° in 0.15 M KCl. One additional titration in the presence of chitotriose in 0.50 M KCl was performed. In order to minimize hydrolysis of the polysaccharides the titrations usually were carried out rapidly with the lysozyme–substrate solutions in the pH range of maximum activity for less than 0.5 hr. This was not true for glycol chitin because of the long mixing time due to the high viscosity of the solution.

The resulting difference data were fitted with the sum of three positive and two negative ideal titration curves in a manner similar to that described previously (Parsons and Raftery, 1972a) for reasons discussed under Results. The pH dependency for the dissociation constant of the trimer–Asp-52 ester derivative complex was determined by an ultraviolet (uv) difference spectrum method (Dahlquist *et al.*, 1966) at 22° in 0.01 M citrate phosphate mixed buffers made up to 0.15 M potassium ion with KCl.

Results

Difference Curve Shape. Figures 1, 2, 3, and 4 show the difference pH data obtained for the titrations performed in the presence of various inhibitors at 25° in 0.15 M KCl. We first will discuss the curve in Figure 3 which was obtained in the presence of chitotriose. The shape of the difference has changed from that found for the uncomplexed proteins. When attempts were made to fit Figure 3 with three titration constants, no values could be found which would give a good fit. In the best fit possible the data points between pH 3.5 to 5.0 fell significantly below the calculated line; that is, the peak around pH 4.5 was flatter than could be accounted for. The poor fit is not illustrated here. A similar titration in 0.5

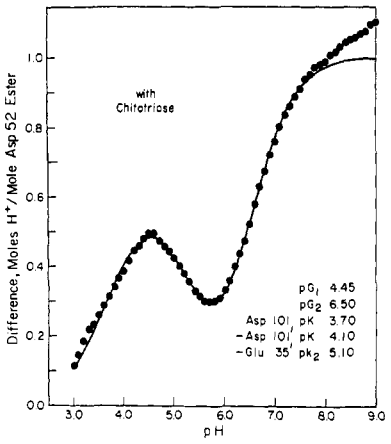

FIGURE 3: The difference pH titration in the presence of 2.5 mg of chitotriose at 25° in 0.15 M KCl. The data require five titration constants for a close fit. The numbers pG_1, pG_2, and pk_2 have the same meaning as in the preceding papers. The pK value for Asp-101′ is for the ester derivative. Binding subsites ABC are filled.

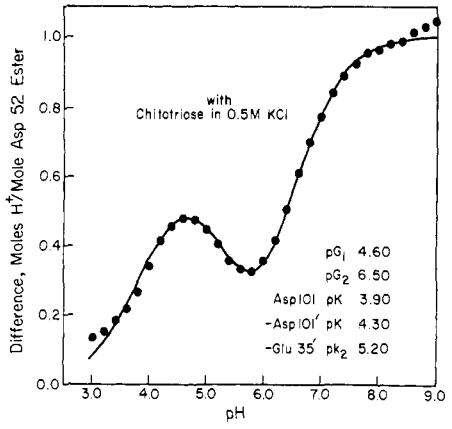

FIGURE 5: The difference pH titration in the presence of 2.5 mg of chitotriose at 25° in 0.50 M KCl. The data require five titration constants for a close fit. The numbers pG_1, pG_2, and pk_2 have the same meaning as in the preceding papers. The pK value for Asp-101′ is for the ester derivative.

M KCl (Figure 5) suffered the same ill fit. From this result and from our previous results, we have no reason to believe that the ill fit was due to a variable electrostatic potential in the region of the active site over this pH range.

Some difficulties are expected in these titrations because we perturbed an area close to the primary binding region, subsite C, when we esterified Asp-52. Thus, an inhibitor, such as chitotriose, may find steric hindrance to its proper binding. This does not matter in the difference titration unless part of the chitotriose molecule interacts with some other titratable group. If this is the case, then a nonelectrostatic mechanism for transmitting the perturbation of Asp-52 to some distant group in the ester derivative is created. In fact, we know from the X-ray crystallographic structure of the chitotriose–lysozyme complex that the chitotriose molecule forms two hydrogen bonds with aspartic acid residue 101 at the top of the cleft (see Figure 8). Dahlquist and Raftery (1968) have assigned a pK of 4.2 to this residue in the free enzyme and a pK of 3.6 to it in the chitotriose complex. If Asp-101 does not have a pK of 3.6 in the chitotriose–Asp-52 ester derivative complex, then the distortion of the difference data in Figure 3 between pH 3.5 and 5.0 might be explained.

pH Dependence of the Association of NAG_3. We can check this possibility by determining the pH dependence of the dissociation constant for the derivative complex. In native lysozyme, the lowering of the Asp-101 pK upon complex formation results in an equal increase in pK_s through the pH range 3.5–4.5 (Dahlquist *et al.*, 1966). This is shown by the dashed line in Figure 6. If the same pK change occurs when chitotriose binds to the ester derivative a similar break in the binding curve will occur. Figure 6 shows that there is little dependency of pK_s on pH over the pH range 3–6 for the derivative complex. Thus, the pK of Asp-101, about 4.2, is nearly the same in both the free Asp-52 derivative and in the chitotriose complex.

Fitting the Difference Data. The results of Figure 6 confirm that a total of five titration constants is the minimum number which will be required to fit Figure 3. Equation 1 describes

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

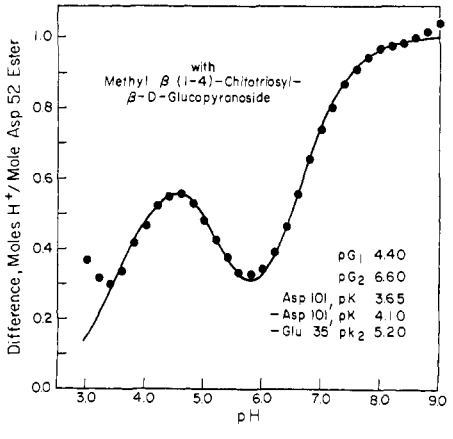

FIGURE 4: The difference pH titration in the presence of 20 mg of methyl β(1–4)-chitotriosyl-β-D-glucopyranoside at 25° in 0.15 M KCl. The data require five titration constants for a close fit. The numbers pG_1, pG_2, and pk_2 have the same meaning as in the preceding papers. The pK value for Asp-101′ is for the ester derivative. Binding subsites ABCD are filled.

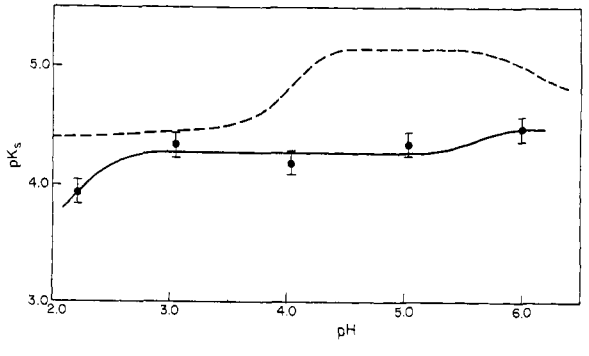

FIGURE 6: The negative logarithm of the dissociation constant for the chitotriose–Asp-52 ester derivative complex *vs.* pH as determined by a uv difference spectrum method at 22° in 0.01 M citrate–phosphate buffers made up to 0.15 M potassium ion with KCl. The dashed line is the binding curve for native lysozyme.

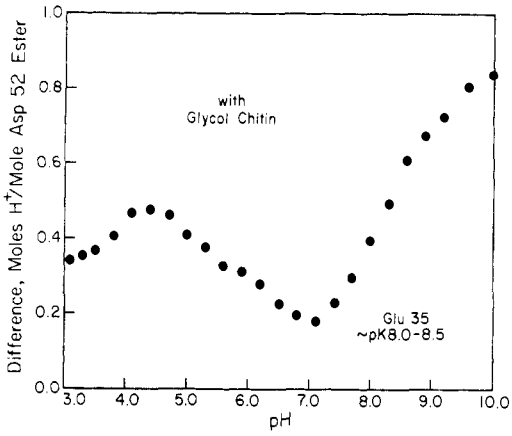

FIGURE 7: The difference pH titration in the presence of 15 mg of glycol chitin at 25° in 0.15 M KCl. Binding subsites ABCDEF are filled. The apparent pK of Glu-35 is about 8.0 to 8.5.

the fitting procedure. Here G_1 and G_2 are the "titration" constants for the strongly interacting Asp-52 and Glu-35 carboxyls 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 Glu-35′ in the ester derivative, K is the dissociation constant for Asp-101 in the native enzyme complex, K' is the dissociation constant for Asp-101′ in the ester derivative complex, and $\Delta\bar{h}$ is the differential proton uptake. The constants determined here are apparent constants containing activity coefficients.

The titration of Asp-101 must have a pK of about 3.6 and that of Asp-101′ a pK' of about 4.2. Figure 6 also tells us that the Glu-35′ pk_2 will be changed little from 5.2 in the complex. Furthermore, we anticipate from other results discussed earlier that the Asp-52 titration constant will be unchanged from the value 4.4 determined in the free enzyme and that the Glu-35 titration constant will be about 6.5. With these five initial guesses we proceeded to fit the difference data in Figure 3. Only minor adjustments were required to obtain a good fit up to pH 8. The values are 4.45 for pG_1, 6.50 for pG_2, 3.70 for the pK of Asp-101, 4.10 for the pK of Asp-101′ in the derivative, and 5.10 for pk_2 of Glu-35′ in the derivative. The titration constants G_1, G_2, and k_2 have the same meaning and relationships to Asp-52 and Glu-35 as discussed in the preceding papers. Because we have shown previously that Asp-101 does not interact strongly in any direct manner with the catalytic carboxyls, K and K' can be taken as true apparent dissociation constants for Asp-101 and Asp-101′, respectively.

The difference data in Figures 1, 2, and 4 also were fitted with five titration constants in a similar way. The pK value for Asp-101 in each of these figures was taken to be variable between 3.6 and 4.2 with the pK' value for Asp-101′ taken to be about 4.2. In general, the data between pH 4 and 8 were given the most weight in fitting. The determined constants in each case are listed on the figure.

At the pH extremes the difference data deviate considerably from the calculated lines in Figures 1, 2, 3, and 4. The deviation at high pH always occurs in the same direction and probably reflects a group which has a lower pK in native lysozyme than in the ester derivative when in the presence of inhibitors. The deviation at low pH is probably experimental error caused by the hurried titration procedure, although the possibility of a further perturbation at very low pH is not excluded.

Figure 7 shows the difference data obtained from the glycol chitin titrations. The data are not very good for several reasons. First, some portion of glycol chitin contains a titratable group which is not removed by MB-1 resin. The titration of this group occurred between pH 6 and 8 and consumed half the amount of titrant consumed by the protein. It was for this reason that aliquots of a stock solution of glycol chitin were used. Second, the titration solution was quite viscous, thus hampering thorough equilibration. Third, such a large substrate can be expected to interact with many titratable groups in native lysozyme in ways not possible in the ester derivative complex. Accordingly, no attempt was made to calculate a difference curve fitting Figure 7.

Nevertheless, the general shape of the data, with two rising limbs at high and low pH and a descending limb between them, is similar to the previous difference curves. The titration occurring at high pH spans 0.7 mole of H^+ per mole of Asp-52 ester and reasonably can be identified as due to Glu-35. Its approximate pK is 8.0–8.5. No more information can be obtained from Figure 7 except that the points are consistent with an Asp-52 pK of about 4.

Discussion

Binding of Inhibitors and Substrate. The binding orientations for the inhibitors and substrate used here are well established by X-ray crystallographic (Blake *et al.*, 1967) and nuclear magnetic resonance results (Raftery *et al.*, 1968b). Figure 8 illustrates the subsite positions assigned to each inhibitor relative to Asp-52, Glu-35, and Asp-101. Subsites E and F are inferred extensions of the binding cleft to which high molecular weight substrates bind.

On the basis of known binding constants (Dahlquist *et al.*, 1966; Pollock *et al.*, 1968; Raftery *et al.*, 1968a) the concentrations of inhibitors utilized here are sufficient to achieve near saturation of native lysozyme. Glycol chitin should bind tightly also. Based on a molecular weight of 264 for each residue, there were 27 residues of glycol chitin per molecule of lysozyme, a ratio surely sufficient for saturation of the enzyme. The Asp-52 ester derivative also will be saturated by chitotriose under these conditions (Figure 6). No information is available on the binding of the other inhibitors or glycol chitin to the derivative.

All the oligomeric inhibitors utilized here are slowly hydrolyzed by lysozyme. However, the hydrolysis requires incubation for many hours at elevated temperature and high "inhibitor" levels (Zehavi *et al.*, 1968a,b; Rand-Meir *et al.*, 1969; Rupley, 1967). Little hydrolysis of the "inhibitors" will have occurred under the conditions of these titrations. Significant reaction of glycol chitin will occur (Hamaguchi *et al.*, 1960). However, the occurrence of transglycosylation will help to maintain a high molecular weight glycol chitin. But even after much cleavage the products still will be of such a high molecular weight that they should be capable of filling the entire lysozyme binding cleft.

Ionization Constants. If we assume that the value of pk_2 determined for Glu-35′ in the various inhibitor–derivative complexes is equal to the microconstant for the Glu-35 ionization in native lysozyme when Asp-52 is protonated, we can calculate all four microconstants from the two titration constants G_1 and G_2 as was done in the two preceding papers. This assumption is more questionable in this instance, though, because we know that inhibitors do not bind in quite the same way to the derivative as they do to lysozyme. However, since the computed values of pk_1 and pk_3 differ only slightly from the measured titration constants, a moderate error in pk_2

TABLE I: The Microconstants for Asp-52 and Glu-35 and the Dissociation Constants for Asp-101 and Asp-101′ in Some Complexes.[a]

Ionization Step[b]		Inhibitor or Substrate Complex[c]				
		NAG-βCH_3	NAG_2-βCH_3	NAG_3	NAG_3-Glu-βCH_3	Glycol Chitin
pk_1 (Asp-52)	4.43	4.43	4.47	4.55	4.47	
pk_2 (Glu-35′)	5.22	5.10	5.20	5.10	5.20	
pk_3 (Glu-35)	6.01	6.42	6.53	6.40	6.53	8.0–8.5
pk_4 (Asp-52)	5.22	5.75	5.80	5.85	5.80	
Asp-101	[4.2][d]	4.3	3.9	3.7	3.65	
Asp-101′	[4.2]	4.1	4.1	4.1	4.1	

[a] Determined at 25° in 0.15 M KCl. [b] The microsteps, pk, are identified in Parsons and Raftery, 1972a, Figure 4. Asp-101′ is the residue in the derivative. [c] The constants are listed for each ionization under each sugar. [d] Value taken from Dahlquist *et al.*, 1966.

will have little consequence. The calculations of the microconstants have been carried out and are listed in Table I. Also listed are the dissociation constants found for Asp-101 and Asp-101′.

Glutamic 35. Microconstant pk_3, which dominates the ionization of Glu-35, shows the expected 0.5-unit increase when subsite C in Figure 8 is filled by NAG-βCH_3, NAG_2-βCH_3, or NAG_3. Microconstant pk_2 does not show this increase. The 0.5-unit increase in pk_3 could be due to an enhanced electrostatic interaction between ionized Asp-52 and Glu-35 as a result of displacing water from the cleft when inhibitor binds. This mechanism then would give no similar increase in pk_2 when an inhibitor bound. Alternatively, the 0.5-unit increase could result from a specific conformational change in the Glu-35 environment which is induced by binding but which does not occur in the derivative because of the perturbed binding.

It is somewhat surprising that Glu-35 shows no further change in pk_3 when subsite D as well as C is filled. This result confirms a kinetic observation on the hydrolysis of nitrophenyl β(1–4)-2-acetamido-2-deoxyglucopyranosyl-β-glucopyranoside, NAG-Glu-β-$C_6H_4NO_3$, which has been shown to bind in subsites CDE. The Michaelis constant for this substrate depends on a group of about pK 5.8 in the free enzyme which increases to about 6.3 in the complex (Rand-Meir *et al.*, 1969). This also would seem to indicate that the dissociation constant of Glu-35 is not sensitive to whether a methyl group or a nitrophenyl group occupies subsite E.

In the presence of the cleft-filling substrate glycol chitin pk_3 for Glu-35 jumps to 8.0–8.5. This is an important observation which correlates well with the apparent value of 8.7 for the hydrolysis of *Micrococcus lysodeikticus* under saturating conditions (Parsons *et al.*, 1969). This should dispel the last doubts (Neuberger and Wilson, 1967) that Glu-35 is the exclusive general acid functioning in the hydrolytic mechanism.

Aspartic 52. Aspartic acid residue 52 appears to undergo no shift in its pk_1 upon complexation. At first thought, the lack of change in pk_1 when subsites C and D are filled is surprising. As was discussed in the preceding paper (Parsons and Raftery, 1972b), this is more understandable if Asp-52 is solvated primarily by its surrounding protein structure. This conclusion correlates well with some recent X-ray crystallographic studies by Beddell *et al.*, 1970, on a lysozyme–NAG–Glu complex. They observed that the Asp-52β carboxylate *in the inhibitor complex* was hydrogen bonded to Asn-46, Asn-59, and to the C-2 hydroxyl of the glucose ring in subsite D. In forming the complex, probably a single water molecule is displaced from Asp-52 and then replaced by the C-2 glucose hydroxyl. Thus the Asp-52 side chain remains equally solvated in the complex, its pk_1 is unchanged, and it is still in a somewhat hydrophobic environment similar to that of the free enzyme. The hydrolytically observed pK values for small molecular weight substrates have generally not been determined accurately enough for an exact comparison with the properties of Asp-52 determined in this work. One can say, though, that the pH–activity profiles for small substrates are all consistent with a value for Asp-52 close to 4.4 (Rand-Meir *et al.*, 1969; Osawa and Nakazawa, 1966; Rupley, 1967; Davies *et al.*, 1969). Because of the constancy of pk_1 and the shape of Figure 7, the results indicate that Asp-52 probably has a dissociation constant of about 4.4 in the presence of high molecular weight substrates. The observed pK for Asp-52 in the hydrolysis of *Micrococcus lysodeikticus* under saturating conditions is 4.5 (Parsons *et al.*, 1969). The apparent change in pk_4 when inhibitors bind

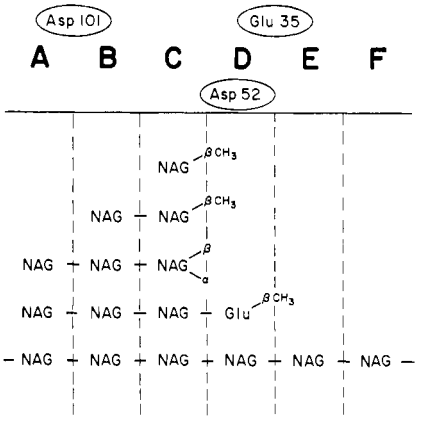

FIGURE 8: Scheme for the relative modes of association of lysozyme with various inhibitors and substrates. Binding subsites A through F are those suggested by Blake *et al.*, 1967. The positions of the three carboxyls known to interact with bound saccharides are shown at the top. Hydrolysis of substrates occurs between subsites D and E.

cannot be analyzed without knowledge of the mechanism of the 0.5-unit effect on pk_3. It could be the result of an increased electrostatic interaction with Glu-35 or merely the spurious result of an incorrect value of pk_2 due to perturbed binding as discussed above.

Aspartic 101. The pK behavior of aspartic acid residue 101 in native lysozyme follows the expected pattern. NAG-βCH_3 does not interact with the residue. Thus the pK of Asp-101 in this complex is 4.3, about the value in the free enzyme. NAG_2-βCH_3 is predicted from the crystallographic structure to form a hydrogen bond between the 6-hydroxyl of ring B and the Asp-101 carboxylate, thus lowering the pK. Table I indicates that this results in a pK of 3.9. NAG_3 forms the same hydrogen bond plus one more from the ring A acetamido NH to Asp-101. A further lowering of pK occurs to 3.7, a value in good agreement with the previously determined number. NAG_3-Glu-βCH_3 should interact with Asp-101 in the same manner if it binds as expected to subsites ABCD. The observed pK of 3.65 for Asp-101 confirms this.

It is of interest that Asp-101′ in the derivative does not form hydrogen bonds to the inhibitors even though it is most likely that the inhibitors are bound in the expected subsites for the following reasons. NAG_3 gives the uv difference spectrum; NAG_3 certainly will not bind strongly to subsites CDE, and it is unlikely that NAG_3 would bind as well in a totally different position in the derivative as its binds to native lysozyme at low pH. It is probable that the binding orientation of NAG_3 is distorted in the derivative just enough to break the hydrogen bonds to Asp-101′. Thus this hydrogen-bonding situation appears to be very sensitive to an exact orientation of the inhibitor or substrate.

Conclusion

Except for generalized charge effects from the rest of the molecule, the titration behavior of Asp-52 appears not to change over a wide range of ionic strengths, both in the free enzyme and in the NAG_3 complex. The same can be said for Glu-35. Interestingly, Asp-101 and Asp-101′ exhibit an increase in their pK's in 0.50 M KCl similar to the increase for Asp-52 (Figure 5), a consequence of the generalized electrostatic potential. Thus the environment around the two catalytic carboxyls as reflected in their ionizations, and therefore the conformation of the active site, appears to be stable over the salt range 0.02 M–0.50 M KCl. The highest concentration of salt which was studied is but moderately below 0.75 M NaCl in which lysozyme crystals are stable (Praissman and Rupley, 1968). There is no indication then that the active site structures in the crystal and in solution differ as a direct result of ionic strength effects.

Our models in this and the preceding two papers (Parsons and Raftery, 1972a,b) for analyzing the ionizations of Asp-52 and Glu-35 have been the simplest possible. Their apparent adequacy is indeed somewhat surprising. However, as discussed in the first paper of this series, lysozyme is probably an ideal protein in which to attempt such an extensive analysis because of chloride ion binding and the partial buried isolation of the two catalytic groups.

We have shown that the titration behavior of the three carboxyls in lysozyme which interact with inhibitors and substrates correlates well with other known catalytic, binding, and magnetic effects. Our deductions about the nature of the Asp-52, Glu-35, and Asp-101 ionizations, and the actual adequacy of our models, await other confirmatory or modificatory data. Not all of the functionally important titratable groups necessarily have been identified and characterized. There are unexplained effects which have been observed (Rand-Meir *et al.*, 1969; Davies *et al.*, 1969). Although great progress has been made toward understanding lysozyme, the catalog of structural and physical information is not yet complete.

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