THE CATALYTIC SITE OF LYSOZYME

Thesis
by
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ABSTRACT

Proton magnetic resonance techniques have used to study the association of simple cyclohexane derivatives to the primary binding subsite of lysozyme in solution. The results agree in detail with crystallographic deductions about the nature of the important binding interactions in subsite C.

A reagent new to protein modification studies, triethylloxonium fluoroborate, has been shown to selectively esterify carboxyl groups under mild aqueous conditions. Two single ethyl esters of lysozyme were isolated chromatographically in good yield and were characterized. One is enzymatically active and reverts to lysozyme under neutral conditions. The other is enzymatically inactive but still binds the inhibitor chitotriose. This inactive ester was identified as occurring at aspartic acid residue 52 by α-chymotryptic digestion of the oxidized derivative and direct observation of the β-ethyl aspartate released from the modified peptide by carboxypeptidase.

Difference pH titrations of the Asp 52 ester vs. the native lysozyme show a difference in proton uptake due to one group over the pH range 3 to 9. The titration of glutamic acid residue 35 in the derivative is perturbed, though, and from the shape of the difference titration curve the ionization behavior of Asp 52 and Glu 35 in native lysozyme and of Glu 35′ in the derivative can be determined. Four microscopic constants for the interacting Asp 52-Glu 35 system considered as a dibasic acid are calculated for each difference curve.
The macroscopic pK of Asp 52 is 4.5 and that of Glu 35 is 5.9 in 0.15 M KCl at 25°C. The ionization of Asp 52 is very dependent on the ionic strength of the solution in going from 0.02 M to 0.50 M KCl and it is shown that Asp 52 is in an environment only somewhat less hydrophobic than that of Glu 35. The heats of ionization determined over the temperature range 1.6° to 40° give an observed ΔH equal to 3.5 kcal/mole for Asp 52 and 2.9 kcal/mole for Glu 35. When these values are corrected for charge interactions in an approximate way they become more normal. The corrected value for Asp 52 can be interpreted in a manner consistent with hydrogen bonding to Asn 46 and Asn 59.

Lysozyme inhibitors and small substrates are found not to perturb the ionization of Asp 52. The macroscopic pK of Glu 35 increases to 6.5 when binding subsites A through D are filled. The pK of Glu 35 exhibits a dramatic shift to 8–8.5 in the presence of the high molecular weight substrate glycol chitin. In addition, the pK of Asp 101 is determined to be about 4.1 in free lysozyme, about 3.9 when subsite B is filled, and about 3.7 when subsite A is filled. These hydrogen bond mediated pK changes do not occur in the derivative and apparently require a precise binding orientation.
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CHAPTER I

Binding Interactions between Lysozyme Inhibitors and Subsite C of Lysozyme as Determined by Proton Magnetic Resonance

Introduction

The three dimensional structure determination of lysozyme by X-ray analysis methods (1) followed by further structural studies of inhibitor complexes with the enzyme (2) has provided a detailed account of atomic interactions for association of various oligosaccharides with the crystalline enzyme. A groove extending down one side of the macromolecule has been shown to contain a series of six subsites which are capable of interacting with pyranoside rings. This is depicted in Figure 1. Based on the X-ray analysis findings for association of NAG*, NAG-NAG, NAG-NAG-NAG, NAM, and NAG-NAM the scheme for relative binding modes shown in Figure 1 has been proposed for these saccharides. In addition, a proposal for the location of the catalytic site of the enzyme, between sites D and E has been put forward. As a result of this, hydrolysis of larger oligomers of NAG or NAG-NAM would proceed as indicated in the

* NAG, N-acetyl-D-glucosamine; NAG-NAG, chitobiose; NAG-NAG-NAG, chitotriose; NAM, N-acetyl-D-muramic acid; NAG-NAM, N-acetyl-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-muramic acid; ACHol, trans-2-acetamidocyclohexanol; ACH, acetamidocyclohexane; n.m.r., nuclear magnetic resonance; p.m.r., proton magnetic resonance.
Recent chemical evidence (3, 4) has substantiated the proposal for the location of the catalytic site and the mechanism for lysozyme catalysed hydrolysis of a selected substrate has been shown to involve an enzyme bound carbonium ion.

The n.m.r. method which we have used to study the association of inhibitors and substrates with lysozyme has been detailed in several recent publications (5-9). Advantage is taken of the fact that the rates of formation and dissociation of enzyme complexes of the inhibitors and substrates are rapid. When nuclei in the associating small molecules experience a change in environment on becoming bound to the enzyme, chemical shift changes occur. In the fast exchange limit the observed spectrum of the small molecule represents a weighted average of enzyme bound and free species. It has been shown that for the equilibrium

$$E + I \rightleftharpoons EI$$

$$I_o = E_o \delta - K_I$$

where $I_o$ and $E_o$ represent the total concentrations of inhibitor and enzyme, respectively, $\delta$ is the observed chemical shift of a nucleus in the associating small molecule (taking its chemical shift in the absence of enzyme as zero) and $\Delta$ represents the chemical shift of the same nucleus in the enzyme bound state. Thus the parameters $K_I$ (the dissociation constant) and $\Delta$ can be evaluated. This gives a measure of the magnetic environment experienced by a nucleus of the
small molecule when it is associated with the enzyme. This Chapter describes the use of nuclear magnetic resonance to study the association with lysozyme of a variety of inhibitors and substrates, including a substrate whose hydrolysis by the enzyme has been shown to proceed through a carbonium ion intermediate (10). We have demonstrated that the relative modes of binding for each of these inhibitors and substrates as deduced by n.m.r. are in good agreement with the scheme proposed from X-ray crystallography. In addition, we have obtained evidence that the detailed atomic interactions between inhibitors and binding subsite C of lysozyme are as deduced from the crystal structure.
Experimental

Materials. -- N-Acetyl-D-glucosamine (m.p. 203-205°) was purchased from Calbiochem. N-Acetyl-d₃-D-glucosamine was obtained by acetylation of D-glucosamine-HCl (Calbiochem) with acetic anhydride-d₆ (Volk Radiochemical Company (Chicago)) according to published procedures (11). Recrystallization of the resulting N-acetyl(d₃)-α-D-glucopyranose was effected from aqueous-alcohol mixtures (m.p. 203-205°). Methyl-2-acetamido-2-deoxy-α-D-glucopyranoside (m.p. 188°) was synthesized as previously reported (12) and purified by chromatography on a charcoal-Celite column. Methyl-2-acetamido-2-deoxy-β-D-glucopyranoside (m.p. 204-205°) was synthesized (13) from D-glucosamine. Synthesis and resolution of D-trans-2-d₃-acetamidocyclohexanol (m.p. 153-154°) and L-trans-2-d₃-acetamidocyclohexanol (m.p. 154-155°) was accomplished with acetic anhydride-d₆ as previously described (14). D₃-Acetamidocyclohexane (m.p. 104-105°) was made by d₃-acetylation of cyclohexylamine. Methyl-β-chitobioside (m.p. 287-288°) and methyl-β-chitotrioside (m.p. 306-310°, d) were prepared by Koenigs-Knorr synthesis from peracetyl sugars. Chitin oligosaccharides were prepared by partial acid hydrolysis (15) of chitin (Sigma) by gel filtration on Bio Gel, P-2 (16). NAG glucosides were prepared by utilizing the transferase activity of lysozyme (11). Lysozyme was obtained from Sigma.
Methods. -- The 60-MHz p.m.r. spectra were recorded on a Varian A-60A spectrometer at a probe temperature of 40°. A Varian HA-100 spectrometer, operating in frequency sweep mode, was used for the 100-MHz p.m.r. spectra, which were measured at 30°. The water resonance was used as a lock signal for the studies in H₂O while a capillary of TMS was used in the studies conducted in D₂O.

The chemical shifts for the 100-MHz spectra were obtained by counting the frequency difference between the manual oscillator and the sweep oscillator on the HA-100 spectrometer with a Hewlett Packard model 5212A counter. All chemical shifts were measured relative to an internal standard of acetone or methanol (0.5%). In each case the chemical shifts were measured at least three times with a standard deviation of 0.04 cps or less. Data were analyzed by methods of least squares. Two sigma errors are given.

Enzyme concentrations of samples used for the p.m.r. measurements were determined from ultraviolet absorbance at 280 mµ of a 25-µl aliquot, after dilution to 5 ml with water, with the use of the known extinction coefficient (13) for lysozyme.

Solutions for competition experiments were prepared by dividing a solution of 50 mg/ml lysozyme and 3.17 × 10⁻² M NAG in 0.1 M citrate pH 5.5 into two portions. The competing inhibitor was then dissolved in one portion according to the highest concentration that was to be studied. Various ratios of the two enzyme solutions were mixed to achieve lower concentrations of competitor. The position of the acetamido resonance of α-NAG was recorded for each
concentration of competitor. Use of deuteroacetamido competitors was necessary in order to avoid interference with the $\alpha$-NAG resonance.
Results

Chemical shift parameters for the acetamido methyl protons of NAG and the β-(1-4)-linked di-, tri-, and tetrasaccharides derived from it are shown in Table 1. In each case the resonance to highest field corresponds to the acetamido methyl group at the reducing end of the molecule. Similar parameters for the methyl-β-glycosides are given in the table and, in addition, the chemical shifts of the glycosidic methyl groups are shown.

In Table 2 the chemical shift changes obtained for various inhibitors upon complexation with lysozyme under various conditions are shown. It is evident that for all the saccharides with free reducing groups the acetamido methyl resonance at the reducing end of the molecule undergoes a large chemical shift to higher field upon binding to the enzyme. The methyl-β-glycosides upon complex formation are seen to display similar chemical shift changes in only the acetamido methyl resonances proximal to the glycosidic methyl group. In addition, the glycosidic methyl group resonances of all glycosides undergo a smaller chemical shift to lower field in the presence of the enzyme.

A quantitative analysis of the chemical shift data obtained for the NAG-lysozyme system is complicated by the mutarotation equilibrium between the α and β anomers of the inhibitor. To decide whether the α- and β-anomeric forms of NAG compete for the same sites on the enzyme surface, a sample of crystalline
N-acetyl(\text{d}_6)-\alpha-D-glucopyranose (m. p. 203-205°) was added to an equilibrated mixture of NAG and lysozyme and the p. m. r. spectrum was recorded at 60 MHz within 2 min after mixing. As seen in Figure 2, the added \( \alpha-(\text{d}_6) \) anomer (although not observable) decreased the observed chemical shifts (and therefore the percentage bound) of both \( \alpha- \) and \( \beta-NAG \). Table 3 shows the quantitative effects observed. As can be seen from Table 3, both anomers were approximately equally affected by the added \( \alpha-(\text{d}_6) \) anomer. The conclusion from this experiment is that \( \alpha-NAG \) and \( \beta-NAG \) do compete for the same sites on the enzyme. The separate binding constants and magnetic environments for the two anomers have been determined (6). The two are found to bind in different orientations with slightly different binding constants.

In addition to studies of binding of oligosaccharides and glycosides containing only NAG residues substrates which contain one NAG and one glucose residue have been studied. Compounds 14 and 15 in Figure 5 are in this category. These compounds have been shown to be hydrolysed specifically at the glucosidic bond (17) by a carbonium ion mechanism (10, 18). In view of the suggested location of the catalytic site between subsites D and E (2) from X-ray analysis evidence and subsequent chemical modification studies which confirm this suggestion (3, 4) it was of interest to subject these compounds to study by the n. m. r. method. It was found that the acetamido methyl group underwent the anticipated chemical shift due to binding in subsite C. In addition, the dissociation constant obtained
\( K_S = 2 \times 10^{-2} M \) was in agreement with the \( K_M \) value determined by kinetic studies of lysozyme catalyzed glycosidic bond cleavage of compound 14.

The results obtained from these n.m.r. studies of complexation of the enzyme with a series of saccharides allow definition of their relative modes of association. Since the monosaccharide NAG was shown to bind only to subsite C, albeit in two competing modes depending on anomeric form (5, 6), it was of interest to attempt to define the specific features of the sugar which are necessary for association with this subsite. To that end it was found desirable to extend the applicability of the n.m.r. method in studying compounds 6, 7, and 8 in Figure 5.

Direct chemical shift measurements are not accurate for weakly associating enzyme inhibitors or for inhibitors experiencing an only slightly different magnetic environment on the enzyme surface. These limitations can sometimes be alleviated by the use of competition measurements. If an inhibitor competitive for subsite C is added to a solution of \( \beta \)-methyl-NAG and lysozyme the observed chemical shift of the NAG residue will decrease (5). This behavior is easily quantitated and in the simplest case gives, after good approximations to linear form,

\[
C_0 = \frac{K_C}{K_I} E_o \Delta I \left( \frac{1}{\delta_I} - K_C - \frac{K_C}{K_I} I_0 \right)
\]  

(2)
where $C_0$ represents the total added competitor concentration, $K_C$ is the dissociation constant of the EC complex and the other symbols have been defined above. The ratio of the slope of Equation (2) to that of a similar plot of $I_0$ vs. $1/\delta_1$ obtained under identical conditions but without added competing inhibitor is equal to $K_C/K_I$. Two such experiments are shown in Figure 3.

Varying concentrations of $\text{d}_\text{a}$-acetamidocyclohexane or pure $l$-trans-$2$-$\text{d}_\text{a}$-acetamidocyclohexanol (14, 19) were competed against NAG. The poorly binding $l$ isomer was shown to be free of the much more strongly binding $d$ isomer by the identities of its melting point and absolute rotation to those of demonstrably pure $d$ isomer. The purity of the $d$ isomer can be observed directly since lysozyme splits the resonances of the $d$ and $l$ forms, leaving the $l$ resonance sharp and nearly unshifted. Thus Figure 3 shows that acetamidocyclohexane and $l$-2-trans-acetamidocyclohexanol are equally poor competitors for subsite C binding and have dissociation constants of $12.6 \pm 2.7 \times 10^{-2}$ molar and $15.7 \pm 2.4 \times 10^{-2}$ molar, respectively.

Not determined thus far is $\Delta_I$. A plot of $I_0$ vs. $1/\delta_1$ for inhibitors which undergo small poorly measured shifts will not determine $\Delta_I$ adequately. However, by including the value of $K_I$ obtained from the competition experiment as a data point on the $I_0$ axis, the slope, and thus $\Delta_I$, is much better determined. Such a graph for pure $l$-trans-2-acetamidocyclohexanol, shown in Figure 4, yields $\Delta_l$ equal to $0.22 \pm 0.06$ ppm. A similar plot for acetamidocyclohexane gives $\Delta$ equal to $0.34 \pm 0.07$ ppm.
An analogous situation arises when direct measurements are made on the chemical shifts of two competitive molecules always held in equal concentration. Such situations are those of $\text{dl}$ optical isomers and $\alpha$ and $\beta$ anomers of some sugars. The equation governing this (6) may be expressed as

$$
\ell_0 = \Delta \ell_0 E_0 \frac{K_{d\ell}}{K_\ell} \frac{1}{\delta_\ell} - K_{d\ell} - \frac{K_{d\ell}}{K_\ell} E_0
$$

(3)

where $d$ and $l$ stand for the optical isomers and $K_{d\ell}$ is a reduced dissociation constant for the $d\ell$ mixture equal to $K_dK_\ell/(K_d + K_\ell)$. Provided that the last term in Equation (3) is relatively small, a plot of the $d$ and $l$ inverse chemical shifts vs. their respective equal concentrations will have the same intercept in a manner identical with the $\alpha$ and $\beta$ anomers of NAG (6).

For $d\ell$-trans-2-acetamidocyclohexanol the intercept was well determined by the $d$ isomer. After including it as a data point for the $l$ isomer and utilizing the value of $K_\ell$ found from the competition experiment, we calculate from Equation (3) a $\Delta \ell$ value of $0.23 \pm 0.07$ ppm (Figure 4), in good agreement with the value determined from pure $l$. The $d$ isomer, both in $d\ell$ mixture and pure, gives a dissociation constant of $0.95 \pm 0.16 \times 10^{-2}$ molar and a $\Delta$ value of $0.91 \pm 0.03$ ppm.
Discussion

Previous studies, employing ultraviolet spectroscopic methods, on the association of $\beta$-(1-4)-linked oligosaccharides of 2-acetamido-2-deoxy-D-glucopyranose (20) have allowed calculation of the dissociation constants for the monosaccharide through the hexasaccharide in association with the enzyme. In that study it was shown that binding strength increased with increasing chain length up to the trisaccharide but that the tetra-, penta-, and hexasaccharides did not appear to bind any more strongly than did the trisaccharide. These results indicated that lysozyme contains three contiguous subsites to which acetamidopyranose rings bind strongly. This interpretation is in agreement with findings (2) employing X-ray analysis techniques to study the association of crystalline lysozyme with 2-acetamido-2-deoxy-D-glucopyranose, chitobiose, and, chitotriose.

The n.m.r. evidence shows that the reducing terminii of all free acetamido sugars studied, except for $\alpha$-NAG, and the glycosidic terminii of all the NAG glycosides studied occupy the same magnetic environment on the enzyme. A binding scheme consistent with these results is shown in Figure 5. All saccharides are shown to occupy the same subsite (subsite C) with other residues occupying subsites A, B, and D. From the data in Table 2 it is possible to assign magnetic parameters to subsites A, B, and C. Acetamido methyl resonances in subsite C are shown to undergo chemical shifts of 0.5-0.8 ppm to higher field, while methoxyl resonances in the same
subsite undergo down field shifts of approximately 0.2 ppm. Acetamido methyl groups in subsite B undergo no change in chemical shift while those occupying subsite A are shifted slightly to lower field (\(\sim 0.08\) ppm).

Since subsite C apparently is dominant in the binding of various inhibitors and substrates it is of interest that the important binding forces in subsite C which determine the scheme be elucidated. Data obtained from studies of \(\alpha\)-NAG, \(\beta\)-NAG, methyl-\(\alpha\)-NAG, acetamidocyclohexane, and the \(d\)- and \(l\)-diastereomers of \textit{trans}-2-acetamidocyclohexanol serve to illustrate that the amide functionality is obviously important. The average magnetic environments experienced in the complexed state by the acetamido groups of \(\beta\)-NAG, methyl-\(\beta\)-NAG, and methyl-\(\alpha\)-NAG are the same, whereas that encountered by \(\alpha\)-NAG is different. This suggests that the \(C_1\) hydroxyl proton of this inhibitor interacts with the enzyme and causes a change in its bound orientation. In the lysozyme crystal, a hydrogen bond is formed between the 1-OH of \(\alpha\)-NAG and the main-chain N-H of residue 109. No interaction occurs between the 1-OH of \(\beta\)-NAG and the crystalline enzyme (2).

The similarity of acetamidocyclohexane and \(l\text{-}\text{trans}\)-2-acetamidocyclohexanol as well as the striking difference from \(d\text{-}\text{trans}\)-2-acetamidocyclohexanol in binding to subsite C is explained as follows. Providing that the ring conformations are similar, the hydroxyl group of one of the optical isomers will be related to lysozyme as the 1-OH in \(\beta\)-NAG while that of the other isomer will
correspond to the 3-OH of NAG. Thus we have a thermodynamically exact test of the relative importance of the β-1-OH and the 3-OH to NAG binding, subject to usual trepidations about models. Theoretical calculations (21) as well as empirical rotational correlations (22) indicate that the absolute configuration of the d optical isomer corresponds to the 3-OH of NAG. Thus the 3-OH of NAG contributes a significant part of subsite C binding energy while the β-1-OH contributes nothing, a conclusion in agreement with X-ray crystallographic evidence (2). In concert with this binding contribution, the 3-OH orients the inhibitor in the subsite such that a much greater chemical shift is obtained. The fact that Δ for d-ACHol is substantially different from Δ for β-NAG suggests that there is at least one more important orienting group for subsite C interactions. The X-ray analysis suggests that this is the 6-OH of NAG (2). It should be noted that the additional interaction(s) does not contribute to the binding strength as all NAG derivatives, in fact, bind more poorly than d-ACHol. Table 4 summarizes the results obtained with a series of NAG derivatives and related compounds.

In conclusion, the use of a simple application of n.m.r. to study the binding of substrates and inhibitors to lysozyme has shown that the binding properties of the enzyme in solution correspond in detail with these properties for crystalline enzyme as determined by X-ray analysis methods. The obvious conclusion is that since certain functional properties in both states are similar the structure of the enzyme should be similar in both states. In addition, the simplicity
and rapidity of the n.m.r. technique allows study of enzyme substrate association (in cases where substrate hydrolysis is slow) and at the same time the method is sufficiently informative to yield a detailed analysis of the association process.
References


Table 1. Chemical Shift Data (Apparent Maxima) for Methyl Groups in Chitin Oligosaccharides and Their Methyl Glycosides *

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( \text{CH}_3-N_1^b )</th>
<th>( \text{CH}_3-N_2^b )</th>
<th>( \text{CH}_3-N_3^b )</th>
<th>( \text{CH}_3-N_4^b )</th>
<th>( \text{OCH}_3^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Acetamido-2-deoxy-D-glucopyranose</td>
<td>17.71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl 2-acetamido-2-deoxy-( \beta )-D-glucopyranoside</td>
<td>18.79</td>
<td></td>
<td></td>
<td></td>
<td>14.72</td>
</tr>
<tr>
<td>Chitobiose</td>
<td>18.35</td>
<td>15.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl ( \beta )-chitobioside</td>
<td>19.51</td>
<td>15.33</td>
<td></td>
<td></td>
<td>13.94</td>
</tr>
<tr>
<td>Chitotriose</td>
<td>18.32</td>
<td>16.28</td>
<td>15.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl ( \beta )-chitotrioside</td>
<td>19.53</td>
<td>16.28</td>
<td>15.90</td>
<td></td>
<td>13.33</td>
</tr>
<tr>
<td>Chitotetraose</td>
<td>18.35</td>
<td>16.54</td>
<td>16.54</td>
<td>15.98</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) The acetamido methyl groups are numbered 1, 2, 3, and 4 beginning at the reducing or glycosidic termini of the molecules. All chemical shifts are in Hz at 100 MHz.

\( ^b \) Values relative to acetone; all chemical shifts to higher field.

\( ^c \) Values relative to methanol; all chemical shifts to lower field.

* Data of F. W. Dahlquist.
Table 2. Chemical Shift Data for Inhibitors and Substrates Complexed with Lysozyme at Various pH and Temperature Values *

<table>
<thead>
<tr>
<th>Compound</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Δ(ppm)\textsuperscript{a}</th>
<th>CH\textsubscript{3}-N\textsubscript{2}\textsuperscript{b}</th>
<th>CH\textsubscript{3}N\textsubscript{3}\textsuperscript{b}</th>
<th>-OCH\textsubscript{3}\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-β-NAG</td>
<td>31</td>
<td>4.9-5.4</td>
<td>0.54±0.04</td>
<td>-</td>
<td>-</td>
<td>0.17±0.03</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>&quot;</td>
<td>0.51±0.03</td>
<td>-</td>
<td>-</td>
<td>0.16±0.05</td>
</tr>
<tr>
<td>Chitobiose</td>
<td>45</td>
<td>&quot;</td>
<td>0.57±0.04</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl-β-chitobiose</td>
<td>35</td>
<td>&quot;</td>
<td>0.60±0.05</td>
<td>0</td>
<td>-</td>
<td>0.20±0.05</td>
</tr>
<tr>
<td>Methyl-β-NAG</td>
<td>31</td>
<td>9.7</td>
<td>0.36</td>
<td>-</td>
<td>-</td>
<td>0.61±0.02</td>
</tr>
<tr>
<td>Chitobiose</td>
<td>55</td>
<td>&quot;</td>
<td>0.77±0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methyl-β-chitobioside</td>
<td>55</td>
<td>&quot;</td>
<td>0.80±0.04</td>
<td>0</td>
<td>0.61±0.02</td>
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<tr>
<td>Chitotriose</td>
<td>65</td>
<td>&quot;</td>
<td>0.61\textsuperscript{d}±0.12</td>
<td>0</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Methyl-β-chitotriose</td>
<td>65</td>
<td>&quot;</td>
<td>0.63\textsuperscript{d}</td>
<td>0</td>
<td>0.08</td>
<td>0.19</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The acetamido methyl groups are numbered 1, 2, and 3 beginning at the reducing or glycosidic termini of the inhibitor molecules.

\textsuperscript{b} Values relative to acetone; all chemical shifts to higher field.

\textsuperscript{c} Values relative to methanol; all chemical shifts to lower field.

\textsuperscript{d} Not at fast exchange limit.

\textsuperscript{*} Data of F. W. Dahlquist.
Table 3. Chemical Shift Data for Acetamido Methyl Protons of α-NAG and β-NAG Association with Lysozyme in Absence and Presence of α-NAG(d₃)

Measurements were made at 60 MHz in 0.1 M citrate buffer, at 40°

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Chemical shift relative to acetone</th>
<th>δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>mg/ml</td>
<td>Hz</td>
</tr>
<tr>
<td>α-, β-NAG</td>
<td>0.05</td>
<td>0</td>
<td>10.92</td>
</tr>
<tr>
<td>α-NAG</td>
<td>0.008</td>
<td>50</td>
<td>14.76</td>
</tr>
<tr>
<td>β-NAG</td>
<td>0.008</td>
<td></td>
<td>12.24</td>
</tr>
<tr>
<td>α-NAG</td>
<td>0.008</td>
<td>50</td>
<td>12.54</td>
</tr>
<tr>
<td>β-NAG</td>
<td>0.008</td>
<td></td>
<td>11.50</td>
</tr>
<tr>
<td>α-NAG(d₃)</td>
<td>0.067</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Data for Binding of NAG Various Analogues to Lysozyme

<table>
<thead>
<tr>
<th>INHIBITOR</th>
<th>$K_s$(M)</th>
<th>$\Delta$(p.p.m)</th>
<th>$\Delta$(p.p.m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-NAG</td>
<td>$3.3(\pm0.2) \times 10^{-2}$</td>
<td>0.51 $\pm$ 0.03</td>
<td>—</td>
</tr>
<tr>
<td>$\alpha$-NAG</td>
<td>$1.6(\pm0.1) \times 10^{-2}$</td>
<td>0.68 $\pm$ 0.03</td>
<td>—</td>
</tr>
<tr>
<td>$\beta$-Me-NAG</td>
<td>$3.3(\pm0.5) \times 10^{-2}$</td>
<td>0.54 $\pm$ 0.04</td>
<td>0.17 $\pm$ 0.03</td>
</tr>
<tr>
<td>$\alpha$-Me-NAG</td>
<td>$5.2(\pm0.4) \times 10^{-2}$</td>
<td>0.55 $\pm$ 0.02</td>
<td>0</td>
</tr>
<tr>
<td>ACH</td>
<td>$12.6(\pm2.7) \times 10^{-2}$</td>
<td>0.34 $\pm$ 0.07</td>
<td>—</td>
</tr>
<tr>
<td>ACHol (1)</td>
<td>$15.7(\pm2.4) \times 10^{-2}$</td>
<td>0.22 $\pm$ 0.06</td>
<td>—</td>
</tr>
<tr>
<td>ACHol (d)</td>
<td>$0.55(\pm0.4) \times 10^{-2}$</td>
<td>0.91 $\pm$ 0.03</td>
<td>—</td>
</tr>
</tbody>
</table>

$a$ Upfield from free resonance.

$b$ Downfield from free resonance.
Figure 1. Scheme for the binding modes of several saccharides based on X-ray crystallographic analysis.
Figure 2. Competitive binding to lysozyme of $\alpha$-NAG and $\beta$-NAG. The resonance caused by acetone protons is shown at left. Other resonances shown are (A) the acetamido methyl proton resonance of free NAG; (B) NAG (at mutarotation equilibrium, $1.6 \times 10^{-2}$ M) in the presence of lysozyme; (C) same as in B but with added $\alpha$-NAG(d$_3$) ($6.7 \times 10^{-2}$ M). In this case, the spectrum was recorded immediately after addition of the $\alpha$-deuterio anomер.
Figure 3. The concentration of $d_3$-acetamidocyclohexane (o) or $\ell$-trans-$2-d_3$-acetamidocyclohexanol (•) vs. the inverse chemical shifts of the $\alpha$ anomer of $3.17 \times 10^{-2}$ molar NAG in the presence of $3.2 \times 10^{-3}$ molar lysozyme and 0.1 molar citrate pH 5.5, 31°. The dependence of $I_0$ vs. $1/\delta I$ for $\alpha$NAG (φ) without added competitor under the same conditions is also plotted. The $\beta$ anomer is not shown.
Figure 4. The concentration of $\text{l-trans-2-d_3}$-acetamidocyclo-
hexanol vs. the inverse chemical shift of the $\text{l}$ isomer
in pure $\text{l}$ (●) and in the $\text{dl}$ mixture (○), $3.2 \times 10^{-3}$
molar lysozyme and 0.1 molar citrate pH 5.5, 31°.
The $\text{d}$ isomer in the $\text{dl}$ mixture is not shown. The
ordinate data points with their errors were determined
as described in the text.
Figure 5. Scheme for relative modes of association with lysozyme of various saccharide inhibitors and substrates. Where α- and β-anomeric forms are indicated on a single line, no information on relative binding modes was obtained. Where α- and β-forms are depicted separately (as with α-NAG and β-NAG), different binding modes were elucidated. Where α- and β-forms are shown on the same molecule on two levels, both anomeric forms bind identically. Methyl groups are depicted by \( R \), nitrophenyl groups by \( \text{PhN} \).
CHAPTER II

A New Reagent for Aqueous Esterification of Protein Carboxylates

Introduction

From knowledge of the three-dimensional structure of lysozyme at 2-Å resolution (Blake et al., 1965, 1967 a, b; Phillips, 1967) and knowledge of the structures of lysozyme-inhibitor complexes (Johnson and Phillips, 1965; Blake et al., 1967a) now available, it appears that we should for the first time be close to understanding in detail the mechanism of action of an enzyme. Based on details of a nonproductive lysozyme-chitotriose complex in the crystalline state, it has been suggested as a result of further model building (Blake et al., 1967b; Phillips, 1967) that the amino acid side chains most likely to play an active role in catalysis by the enzyme are the β- and γ-carboxyl groups of aspartic acid residue 52 and glutamic acid residue 35 in the amino acid sequence.

Numerous attempts have been made to identify by chemical means the amino acid side chains which affect catalysis by lysozyme. The approaches used have been reviewed (Jollès, 1964, 1967). In summary, it has been claimed that oxidation (Fraenkel-Conrat, 1950) or reduction (Churchisch, 1962; Imai et al., 1963, Jollès et al., 1964) of disulfide bonds, photooxidation of histidine and aromatic amino acids (Weil et al., 1952), oxidation (Horinishi et al., 1964), iodination (Hartdegen and Rupley, 1964, 1967), or ozonization of
tryptophan (Previero et al., 1966, 1967) residues, acetylation of amino groups (Fraenkel-Conrat, 1950; Geschwind and Li, 1957), and esterification with methanol–HCl of carboxyl groups (Fraenkel-Conrat, 1950) all can cause inactivation of the enzyme.

This chapter presents a new reagent for the selective esterification of protein carboxyl groups. We have sought a method which to the best of our knowledge does not seriously disrupt the secondary or tertiary structures of the enzyme. An important feature of the investigation was the determination of whether inactivation caused by chemical modification of lysozyme resulted from an effect on the binding properties or on the catalytic properties of the enzyme derivatives.
Experimental

**Materials.** -- Lysozyme (Lot No. 96B-8572) was purchased from Sigma Chemical Co. Ninhydrin was a product of the Pierce Chemical Co. N-Acetyl-D-glucosamine was obtained from the California Corp. for Biochemical Research. Chitotriose was prepared from a partial acid hydrolysate of chitin by a gel filtration procedure used in this laboratory (Raftery et al., 1969a). **Micrococcus lysodeikticus** cells were purchased from Miles Laboratories Inc.

Triethyloxonium fluoroborate was synthesized from boron trifluoride etherate and epichlorohydrin (both from Matheson Coleman and Bell Co.) as previously described (Meerwein, 1937) and the product was stored under ether at 0° with desiccation.

The synthesis of $^{14}$C-labeled triethyloxonium fluoroborate was accomplished by incorporation of $[^{14}\text{C}]$diethyl ether into the oxonium salt by exchange. All preparatory manipulations for the exchange were carried out at -80° under a nitrogen atmosphere. A solution of the oxonium salt (0.78 g) in 7.0 ml of methylene chloride containing 1.0 mCi of $[1-^{14}\text{C}]$diethyl ether (74 mg, from New England Nuclear Corp.) was sealed by torch in a heavy-walled glass ampoule. The ampoule was incubated 22 hr in refluxing methylene chloride. The $[^{14}\text{C}]$oxonium salt solution (1 ml) was added to 100 mg of 3, 5-dinitrobenzoic acid in 15 ml of methylene chloride. The mixture was refluxed for 4 hr. After evaporating the methylene chloride, washing
the residue with 5% NaHCO₃, and recrystallizing the residue from EtOH-H₂O, 15 mg of ethyl 3, 5-dinitrobenzoate (mp 82.9-83.7°) resulted with a specific activity of $5.0 \times 10^6$ dpm/mmole. The main bulk of the $^{14}$C oxonium salt was precipitated from methylene chloride solution as an oil by the addition of 20 ml of hexane. The supernatant was poured off and the oil was taken up into a syringe with 0.5 ml of dry acetonitrile. This solution containing about 0.5 g of $^{14}$C oxonium salt was treated with 188 mg of lysozyme in 12 ml of water at pH 4.5 in the manner described below.

**Analytical Methods.** --- Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer after prior hydrolysis of protein samples for 20 hr in constant-boiling HCl, under vacuum, at 105°. Color values of amino acids normally found in hydrolysates of lysozyme were calibrated by standard mixtures (Beckman Co., Spinco Division).

Amino-terminal groups were determined by reaction of protein samples with fluorodinitrobenzene (Sanger, 1945) followed by paper chromatography of dinitrophenylamino acids (Levy, 1954) after acid hydrolysis of the reacted proteins.

Carboxyl ethyl esters were determined (a) by converting them into hydroxamates at pH 7.0 or 9.0 (using 1 M NH₂OH) at 25° for 2 hr. This was followed by dialysis or gel filtration to remove excess hydroxylamine, lyophilization of the protein hydroxamates, and iodine oxidation to nitrite of the hydroxamates followed by determination as described elsewhere (Yasphe et al., 1960); (b) as ethanol, liberated
by mild basic hydrolysis in sealed ampoules, which was quantitatively
determined by gas-liquid partition chromatography using 10% Carbo-
wick 20M on a 60-80 mesh-column, 6 ft, at 90°. Flame ionization
detection and t-butyl alcohol internal standards were used; (c) by
basic saponification of the ester derivatives with standardized NaOH
carried out in a pH-Stat at pH 10.0, at 37°, under nitrogen; or (d)
by coupling of the enzyme ester to glycinamide with a water-soluble
carbodiimide (Hoare and Koshland, 1967) using 5 M guanidinium
hydrochloride as solvent. All protein samples were oxidized with
performic acid (Hirs, 1956) before coupling. Comparison of the
number of glycine residues (estimated by amino acid analysis) coupled
to unmodified lysozyme under similar conditions gave, by difference,
the number of carboxyl esters.

Column chromatographic separations were monitored by (a)
absorbance of effluents at 280 mµ; (b) determination of protein by the
Lowry procedure (Lowry et al., 1951); (c) ninhydrin analysis using a
Beckman-Spinco Model 120B amino acid analyzer while employing a
ninhydrin system previously described (Moore and Stein, 1954); or
(d) manual ninhydrin analysis. Enzymic activity assays on lysozyme
and its derivatives were performed under conditions (Perry et al.,
1965) where relative initial rates could be measured, using M.
lysodeikticus cells as substrate. Activities of various enzyme
preparations using chitotriose as substrate were performed in 0.1 M
citrate buffer (pH 5.5) at 40° for 1 hr. Enzyme concentrations were
3 × 10^{-3} M and chitotriose concentrations were 10^{-2} M. Quantitation
of hydrolysis of chitotriose was effected by separation of aliquots of the hydrolysis mixtures on columns (1.0 x 100 cm) of Bio-Gel P-2 using 0.1 M NaCl as solvent. Concentrations of the mono-, di-, and trisaccharides were determined by the ferricyanide procedure (Park and Johnson, 1949).

Dissociation constants for the binding of chitotriose to lysozyme and lysozyme derivatives were determined by a method previously described (Dahlquist et al., 1966). Dissociation constants for the binding of 2-acetamido-2-deoxy-D-glucopyranose to lysozyme and lysozyme derivatives were determined by proton magnetic resonance methods (Raftery et al., 1968, 1969b).

Chromatography of lysozyme and its derivatives was performed on columns (0.6 x 45 cm) of the carboxylic cation exchanger Bio-Rex 70 (-400 mesh) utilizing two buffer systems. The first system was that previously described (Hirs, 1955) for purification of lysozyme, employing 0.2 M sodium phosphate (pH 7.18). The second buffer system utilized four chambers of a Varigrad (Technion Instruments). The first three chambers contained 0.16 M sodium phosphate (pH 7.2) and the fourth chamber contained 0.20 M sodium phosphate (pH 7.2) which was 0.5 M with respect to KCl. Each chamber contained 40 ml of buffer. Flow rates of 12 ml/hr were employed for such columns and the fraction size was 0.80 ml. All chromatography was performed in jacketed columns which were equilibrated and maintained at 7°.

To determine the specific activity of the 14C derivatives, every odd fraction from an analytical chromatograph was suspended in
15 ml of Bray's scintillation solution (Bray, 1960) containing 4.5% thixotropic gel powder (Packard) and counted for 2 min. In order to compare directly the relative specific activities of the proteins and the benzoate standard, 0.65 mg of the $^{14}$C-labeled ethyl 3,5-dinitrobenzoate derivative was dissolved in 1.00 ml of dioxane. This solution (100 µl) was added to some of the previously counted vials throughout the chromatograph and these were recounted. This small amount of the 3,5-dinitrobenzoate was shown to give very little quenching. The counting efficiency at the beginning of the salt gradient was 69% and smoothly decreased to 63% at the end, as determined by a $[^{14}$C]toluene standard. To calculate the ester content, the known extinction coefficient and molecular weight (Sophianopulos et al., 1962) were used for lysozyme.

**Preparative Methods.**

---

**A. Esterification in ethanol.**

Esterification in ethanol-HCl mixtures was done as described for a similar reaction in methanol-HCl (Fraenkel-Conrat, 1950).

**B. Esterification of lysozyme with triethylxonium fluoroborate.** A typical esterification was performed in the following manner. Lysozyme (188 mg) was dissolved in 15 ml of distilled water and the pH was adjusted to the desired value, pH 4.5, with dilute perchloric acid. Triethylxonium fluoroborate (approximately 0.7 g) was dried under a stream of nitrogen and weighed. Approximately 0.2 g of dry acetonitrile was added to the salt and the resulting solution was weighed. A dry syringe was filled with the solution and the calculated weight containing 0.57 g of reagent was injected into the
vigorously stirred lysozyme solution to give a final concentration of 0.2 M oxonium salt. The pH was maintained by a pH-Stat with addition of about 1 ml of 4 N NaOH. Reaction was over in about 20 min and a slightly cloudy solution resulted. The product was dialyzed against several changes of distilled water and lyophilized. A typical yield was 170 mg of esterified product. Derivatives were prepared in this manner at pH 4.0 and 4.5 with oxonium salt concentrations of 0.1 and 0.2 M. Large preparations involved reaction of 2.00 g of lysozyme in an identical manner on a larger scale.

C. Esterification of lysozyme in the presence of chitotriose. The esterification in the absence of substrate was carried out essentially as already described for reaction at pH 4.5 except that three additions of reagent were made at 20-min intervals. Esterification in the presence of substrate was similarly carried out on lysozyme which was preequilibrated for 2 min in $8 \times 10^{-3}$ M chitotriose solution before addition of the oxonium salt.

D. Esterification of lysozyme with multiple additions of esterification reagents. Lysozyme (500 mg) was dissolved in 40 ml of distilled water and the pH was adjusted to 4.5 with dilute perchloric acid. An acetonitrile solution of 1.52 g of triethylxonium fluoroborate was added and the pH was maintained by addition of 8 N NaOH. The resulting slightly cloudy solution was dialyzed against distilled water for 2 hr after which a 1 ml aliquot was withdrawn for assay of enzymatic activity and the remainder was again subjected to the esterification reaction. The addition of reagent, followed by dialysis
and activity assay, was carried out six times in all and yielded an esterified product which contained an average of 2.6 esters/molecule of enzyme and which had an enzymatic activity equal to 21% that of native lysozyme.

E. Esterification of lysozyme with high concentrations of esterification reagent. Lysozyme (500 mg) was dissolved in 40 ml of distilled water and the pH was adjusted to 4.5 with dilute perchloric acid. Triethylxonium fluoroborate (7.6 g) was added in solid form to give a concentration of 1 M. The pH of the reaction mixture was maintained at 4.5 by addition of 8 M NaOH from a syringe until the pH no longer decreased (approximately 30 min). Some precipitation of protein occurred under these conditions of reaction. The reacted mixture was dialyzed against distilled water and lyophilized.

F. Isolation of esterified derivatives of lysozyme. Preparative chromatography was done on columns (5.0 x 70 cm) of Bio-Rex 70 (-400 mesh) at 7° using 0.2 M sodium phosphate (pH 7.18) as eluting buffer. Loads of 1.5-2.0 g of protein were applied to the columns. Fractions of 30-150 ml were collected using an Isco preparative fraction collector and effluents were monitored with an Isco Model UA-2 ultraviolet analyzer. For precise location of protein peaks fractions were analyzed by reading the absorbance at 280 m\(\mu\) or by the Lowry method (Lowry et al., 1951). After adjusting the pH to either 4 or 5 depending upon the component, rapid desalting of pooled fractions, which were sometimes of the order of 1000 ml, was effected by ultrafiltration at 7° to a small volume (about 40 ml) in an
Amicon Ultrafiltration apparatus (Amicon Corporation) employing a UM-1 membrane followed by dialysis against several changes of distilled water at 2-4°C. The material was then lyophilized.
Results

Modification of Carboxyl Groups in Lysozyme. -- We have prepared an ethyl ester derivative of lysozyme by treatment with dry ethanol–HCl, followed by dialysis and lyophilization. Table 1 shows that as determined by three methods the average ethyl ester content was found to be 5.3 moles/mole of enzyme. Our figure of 5.3 ethyl esters can probably be raised to 6.3 since, as is subsequently shown in later sections of this chapter, lysozyme can form one ethyl ester which is labile and it is likely that this ester was hydrolyzed during these particular ester determinations.

The activity of our ethyl ester derivative when assayed for its ability to lyse *M. lysodeikticus* cells was between 3 and 5% that of native lysozyme. We also tested the ability of this material to bind the inhibitor N-acetyl-D-glucosamine. We have previously shown (Raftery et al., 1968, 1969b) that association of 2-acetamido-2-deoxy-D-glucopyranose and lysozyme can be studied by proton magnetic resonance spectroscopy. It has been shown (Dahlquist and Raftery, 1968) that the α and β anomers of 2-acetamido-2-deoxy-D-glucopyranose while competing for the same binding site on lysozyme do not bind in precisely the same way to that site. The chemical shift undergone by the acetamido methyl group of 2-acetamido-2-deoxy-D-glucopyranose in the presence of lysozyme ethyl ester indicates that 2-acetamido-2-deoxy-D-glucopyranose binds to this modified enzyme. However, in the present instance the resonances of the α and β
anomers were not separated. The chemical shift data are plotted in Figure 1 according to a method previously described (Raftery et al., 1968 and 1969b). The dissociation constant obtained from this plot is $K_S = 8 \times 10^{-2}$ M. The $\Delta$ value, which is discussed later, is 0.58 ppm. Thus association of 2-acetamido-2-deoxy-D-glucopyranose is weaker with the lysozyme ester than with lysozyme where

$$K_\alpha - 2\text{-acetamido-2-deoxy-D-glucopyranose} = 1.8 \times 10^{-2} \text{ M}$$

and

$$K_\beta - 2\text{-acetamido-2-deoxy-D-glucopyranose} = 3.5 \times 10^{-2} \text{ M}$$

(Dahlquist and Raftery, 1968). It was also shown that the ethyl ester derivative of lysozyme had a decreased affinity for chitotriose as determined by an ultraviolet difference spectral technique (Dahlquist et al., 1966) with $K_S = 1.0 \times 10^{-4}$ M, compared with $K_S = 6 \times 10^{-6}$ M for unmodified lysozyme.

For reasons discussed later, we sought a method which would selectively esterify carboxyl groups in the enzyme, which would do so with no irreversible structural alterations in the protein, and which would possibly discriminate among the many carboxyl groups present. Accordingly, triethyl oxonium fluoroborate, a powerful ethylating reagent, was tested. Its reaction with carboxyl groups proceeds according to the pathway in Figure 2. Most of the reagent is hydrolyzed in aqueous solution with the uptake of base.

Specificity of the Esterification of Lysozyme with Triethyl-oxonium Fluoroborate. -- The amino acid composition of lysozyme esterified in 1.0 M oxonium salt at pH 4.5 and containing 5.5 esters as estimated by saponification did not differ from that of unmodified
lysozyme (Table 2), indicating that ethylation of histidine, methionine, cystine, lysine, arginine, serine, threonine, or tyrosine had not occurred. In addition, no new ninhydrin-positive components were visible on the traces obtained from the amino acid analyses columns. Gas-liquid partition chromatography, saponification, and hydroxamate formation all indicated the formation of protein esters. One further check of the specificity of the reaction was performed. In case random O alkylation of amides had occurred, albeit unlikely in aqueous solution, end-group analysis by the Sanger method was carried out on the product containing 5.5 esters to identify any new amino-terminal residues which would have resulted from the spontaneous hydrolysis of O-ethylamides in the primary sequence. By comparison with a simultaneous end-group determination on the parent lysozyme no new amino-terminal residues were detected. Thus it was concluded that the alkylation reaction was specific for esterification of carboxyl groups.

Although complete inactivation of lysozyme by many moderate additions of oxonium salt (Figure 3) was not possible (the lowest activity found under these conditions equaled 21% that of native lysozyme), a strong indication that carboxyl groups are important in catalysis was obtained by performing the esterification in the presence of chitotriose, a known substrate (Wenzel et al., 1962) for the enzyme. Esterification at pH 4.5 with three additions of reagent caused a decrease in activity from 100 to 24%; in the presence of the trisaccharide inactivation by similar treatments was limited since
the product retained 55% of its initial activity. This suggests that partial protection of carboxyl groups which are susceptible to esterification is effected by the binding to lysozyme of chitotriose.

Although 5.5 moles of ethyl ester could be introduced per mole of lysozyme and the relative enzymatic activity could be reduced to 6%, such a procedure was not followed in subsequent work since it was obvious (Figure 3) that the enzymatic activity of lysozyme decreased to 50% of its original value when a limited number of esters (0.8) had been introduced. It seemed, therefore, that some selectivity was operating in the esterification, due perhaps to special reactivity of critical carboxyl groups.

**Esterification of Lysozyme at pH 4.0.** -- Analytical chromatography of the product formed by reaction of lysozyme with 0.1 M triethyloxonium fluoroborate at pH 4 revealed that in addition to unmodified lysozyme, only one main chromatographic component was found. Figure 4B shows a typical chromatographic pattern for such a preparation. Cochromatography of peak I with lysozyme in the same system confirmed their identity. In addition, the enzymatic activity of peak I was found to be 100% that of the parent lysozyme. The activity of the material in peak III was found to be constant across the peak and to be equal to 57% that of the parent lysozyme, if determined immediately after emerging from the column, when assayed for its ability to lyse suspensions of *M. lysodeikticus*.

**Isolation of Component III.** -- Reaction of 2 g of lysozyme in 160 ml of water with triethyloxonium fluoroborate (0.1 M) at pH 4.0
gave 1 g of component III when isolated as described in the Experimental Section at pH 4.0 (except during chromatography). Analytical chromatography of the isolated material showed that it was pure component III.

Determination of the binding constant for association of chitotriose with component III, utilizing a spectrophotometric method (Dahlquist et al., 1966), yielded a dissociation constant, $K_S$, of $3 \times 10^{-4}$ M which showed that relative to unmodified lysozyme ($K_S = 6 \times 10^{-6}$ M) the binding strength was decreased. It seems reasonable to infer that a carboxyl group in or near the three contiguous binding sites on the enzyme surface (Raftery et al., 1969b) was esterified in component III. The amino acid composition of component III is given in Table 2. The ester content of this derivative was shown to be 0.7 ester group/molecule by conversion into the hydroxamate and estimation by the method of Yasphe et al. (1960).

An interesting property of component III is that it reverts to lysozyme on standing in phosphate buffer (pH 7.2) at room temperature over a 48-hr period. Thus the ester formed is labile, possibly due to the properties of the parent carboxylate. Full enzymatic activity was regenerated, relative to lysozyme, as a result of reversion to the parent enzyme. Figure 4C shows the chromatographic position of the reverted product. It occupies a position in the elution diagram identical with that occupied by native enzyme. This is evidence that the ester formed in component III came from a carboxylate group, not an asparagine or a glutamine. This also indicates that component III is
homogeneous.

Esterification of Lysozyme at pH 4.5. -- Analytical chromatography (0.20 M phosphate system) of the product formed by reaction of lysozyme with 0.2 M triethyloxonium fluoroborate at pH 4.5 gave the pattern shown in Figure 5A. It was seen that in addition to a small amount of lysozyme (I), three chromatographic components (II, III, and IV) were observed. Component III was eluted at a position corresponding to that of the major derivative obtained from esterification at pH 4.0. Assay of the peaks I, II, III, and IV showed that component I had a specific enzymatic activity equal to that of lysozyme, while both components II and IV appeared to be inactive. Component III had a specific activity equal to 55% that of native lysozyme, which corresponded to that of the major derivative obtained upon esterification at pH 4.0. The most interesting material in the present instance was component II, since it chromatographed just behind lysozyme and was therefore considered to have a low number of carboxyl ester groups. Its yield in the total mixture was, however, disappointingly low. It was found that if a sample of the reaction mixture containing components I, II, III, and IV was left at room temperature in 0.2 M phosphate, pH 7.18, for 20 hr and then chromatographed, components III and IV reverted to lysozyme and component II, respectively. Figure 5B shows the pattern obtained. Thus the yield of component II could be increased. It seemed reasonable that the most reactive carboxyl at pH 4.0 was esterified in high yield at pH 4.5, but that, in addition, a second carboxyl residue was
esterified, both in lysozyme and in the ester derivative labeled III, giving rise to components II and IV, respectively. It was found that component II contained an ester of expected stability unlike component III.

Where $^{14}$C-labeled oxonium salt was employed for reaction at pH 4.5, analytical chromatography with the gradient system of the $^{14}$C-labeled esters gave the pattern shown in Figure 6. Components I, II, III, and IV were identified by their relative positions and by their enzymatic activities. The low yields of components II and IV were due to the fact that because of the difficulties in handling it, the $[^{14}\text{C}]$ oxonium salt in the lysozyme reaction turned out to be only about 0.15 M. A fifth enzymatically active component, not eluted with 0.20 M sodium phosphate, also appeared but was not investigated. Essentially all of the protein and radioactivity applied to the column was eluted. The radioactivity peaks followed the protein peaks well and it was readily seen that components IV and V contained about twice the counts relative to OD$_{281}$ that components II and III did. Only background levels were found under the lysozyme peak, component I. The calculated specific activities confirmed that component II (inactive) contains 0.96 ester group/molecule, that component III (labile) contains 1.0 ester, and that component IV (inactive and labile) contains 1.9 esters.

**Isolation of Component II.** -- Reaction of 2.0 g of lysozyme with 0.2 M triethyloxonium fluoroborate at pH 4.5 under the conditions outlined in the Experimental Section yielded 1.9 g of total derivative
after dialysis and lyophilization. This was dissolved in 200 ml of 0.2 M phosphate (pH 7.18) and left at room temperature for 20 hr. Chromatography on a preparative column partially separated lysozyme and component II. Material (220 mg) which was predominately component II was isolated at pH 5.0 as described above. The enzymatic activity of isolated component II was shown to be 10-12% that of native lysozyme.

It was of interest to investigate the properties of this preparation using chitin oligosaccharides as substrates and inhibitors. Determination of the dissociation constant of the complex formed between component II and chitotriose gave a value for $K_S$ of $7 \times 10^{-5}$ M compared with $K_S = 6 \times 10^{-6}$ M for lysozyme-chitotriose. This indicated that a carboxyl group at or close to the binding site for chitotriose had been esterified in component II.

Since chitotriose also serves as a substrate for lysozyme the capability of component II to hydrolyze the trisaccharide was also tested. It was shown that treatment of the trisaccharide ($10^{-2}$ M) with component II ($3.0 \times 10^{-3}$ M) at 40° for 1 hr resulted in formation of chitobiose and N-acetyl-D-glucosamine to the extent of only 4% that formed by native enzyme under similar conditions.

Rechromatography of component II on a similar preparative column gave the pattern shown in Figure 7. Assay of the enzymatic activity of each tube showed that the activity was eluted from the column ahead of component II, and was therefore due to contaminating lysozyme. The yield of the rechromatographed component II was
200 mg. The enzymatic activity of rechromatographed component II varied from preparation to preparation and was of the order of 0-1.9% the specific activity of native lysozyme when assayed using *M. lysodeikticus* as substrate. The relative specific activity of one of the better preparations was shown to be less than 0.5% throughout the pH range 2-10. The occasional small amount of activity was probably due to other contaminating single esters which were formed from labile double esters, such as component V in Figure 6, during the incubation at pH 7.2. When the incubation was not carried out and the activity of component II in the effluent of an analytical column was determined, essentially zero activity was found. The amino acid composition of component II is given in Table 2.

In addition to the use of $^{14}$C-labeled reagent to estimate the ethyl ester content of components II, III, and IV as already described, the ethyl ester content of the isolated component II was determined by reaction of the unesterified carboxyl groups with glycinamide in 5 M guanidinium hydrochloride according to Hoare and Koshland (1967), and determination of the number of glycine residues incorporated by amino acid analysis. Comparison with unmodified lysozyme which had been similarly coupled with glycinamide gave by difference the number of carboxyl groups which had been modified by esterification. Table 3 shows the results obtained, and these confirm that component II contains a single ethyl ester which is derived from a carboxyl group.

Another interesting property of component II was that on standing at room temperature at pH 2.0 enzymatic activity was slowly
regenerated to a maximum of 60% that of native lysozyme after 20 hr. Higher values were not obtained due to denaturation at the acid pH. The specific enzymatic activity fell from 60% to lower values on further standing.
Discussion

Virtually all the types of side chains in lysozyme which are susceptible to chemical modification have been implicated in reference to its activity. The apparent non-specificity in the destruction of the lytic ability has doubtless been due partly to the unavailability of a well-defined substrate with which to distinctly separate binding function from catalytic action. In the present instance this difficulty has been overcome by use of chitotriose, a small molecule which serves as a lysozyme substrate, albeit a poor one. Chitotriose has been shown to bind to the enzyme by X-ray analysis methods (Blake et al., 1967a), by difference spectral techniques (Dahlquist et al., 1966; Rupley et al., 1967), by fluorescence techniques (Lehrer and Fasman, 1966, 1967), and by proton magnetic resonance methods (Raftery et al., 1968, 1969b; Dahlquist and Raftery, 1968, 1969). The binding of chitotriose has been shown to involve three contiguous strong binding sites by these methods and the stability of the complex formed suggests that it is a non-productive one. Thus, this association affords a means of studying the strong binding region of the enzyme, while cleavage of chitotriose to chitobiose and 2-acetamido-2-deoxy-D-glucopyranose, presumably at another site on the enzyme, allows estimation of catalytic activity.

Parsons et al. (1969) have demonstrated that the lysine, histidine, and tyrosine residues of lysozyme are not essential to its activity. Whereas modification of various amino acid side chains
resulted in decreased specific activity it was shown that in most cases this reduction resulted from a lowering of the affinity of the enzyme for the substrate or a shift in the optimum pH for hydrolytic activity. All the amino acid functional groups except for carboxyl groups which could act as a general acid in lysozyme catalysis have been shown not to be required.

When lysozyme was treated with reagents which result in the modification of many of the carboxyl groups, most of the enzymatic activity was abolished (Fraenkel-Conrat, 1969; Donovan et al., 1960). Thus, modification by the method of Hoare and Koshland (1966, 1967) with resultant incorporation of seven glycinamide residues or esterification in ethanol-HCl with formation of 5.3 ethyl esters gave products which indicated that carboxyl groups might be critical for the activity of lysozyme. However, such a conclusion, although agreeing with the suggestions of Blake et al. (1967a), is tenuous when based only on the modification of all "available" carboxyls. The inactivation might be due to effects not directly related to modification of the one or two catalytically important (and perhaps not "available") carboxyls.

That the lysozyme derivative containing 5.3 ethyl esters, prepared in ethanol-HCl, has essentially the native conformation is indicated by the binding studies. Both 2-acetamido-2-deoxy-D-glucopyranose, and chitotriose bind to the derivative. The somewhat larger dissociation constants (for β-2-acetamidp-2-deoxy-D-glucopyranose, only twice as large with the derivative as with native enzyme) could be due to direct steric interactions between the ester
groups and the inhibitors. In any case, the retention of substantial inhibitor binding indicates that the tertiary structure of the enzyme in the region of the binding sites was not seriously disrupted by esterification. This is further supported by the fact that 2-acetamido-2-deoxy-D-glucopyranose binds to a site in this derivative that gives rise to an upfield chemical shift (0.58 ppm) in the proton magnetic resonance signal of the acetamido methyl groups similar to that given by β-2-acetamido-2-deoxy-D-glucopyranose, methyl-β-2-acetamido-2-deoxy-D-glucopyranose, and methyl-α-2-acetamido-2-deoxy-D-glucopyranose in association with the native enzyme (0.54 ppm) (Raftery et al., 1969b). It seems, then, that a lysozyme derivative containing a single ester group most likely would be in the native conformation.

We sought a method which would selectively esterify carboxyl groups in aqueous solution under mild conditions. To this end, we tested triethylxonium fluoroborate \([\text{C}_2\text{H}_5)_3\text{O}^+\text{BF}_4^-\]). The capability of this compound to effect ethylation of various nucleophiles is well documented (Booth and Martin, 1949). The reagent discriminates between carboxyl side chains probably on the basis of their pK\(_a\) values (as well as, of course, on the basis of steric requirements) since the oxonium ion is known to attack anions much more rapidly than the corresponding neutral species.

In addition to amino acid analyses and the amino-terminal group determination, many different types of ester analyses were utilized to prove that this powerful alkylator attacks only carboxyl groups.
Agreement was found between methods that in effect detected that carboxyl groups had disappeared and other methods that indicated ester groups present. Treatment of lysozyme with the reagent resulted in a decrease of enzymatic activity. When the trisaccharide, chitotriose, was included in the reaction mixture, some protection against the inactivation was afforded. Thus it seemed that this esterification reagent was ideally suited to investigation of the role played by particular carboxyl groups in lysozyme. It was possible to isolate two esterified derivatives of the enzyme and to determine some of their properties.

A derivative which was formed in a highly preferential esterification at pH 4.0 was shown to contain a single labile ester, to bind chitotriose much less efficiently than native lysozyme (by a factor of 20), but to retain 57% of the specific activity of the enzyme when assayed against *M. lysodeikticus*. Thus, it is probable that the carboxyl group esterified is one which is at or close to the strong binding site (nonproductive complex) for chitotriose rather than at the catalytic site. The lability of this derivative which allows it to revert to lysozyme on standing in solution at pH 7 most likely results from whatever environment makes it the most reactive carboxyl in the enzyme.

The second reactive carboxyl group in lysozyme was esterified in fair yield by reaction with triethyloxonium fluoroborate at pH 4.5. Higher yields of this derivative were isolated by allowing hydrolysis of a labile ester in derivative IV to occur. Thus a doubly esterified
enzyme was converted into a singly esterified species which displayed the stability expected of a normal ethyl ester. This derivative was shown to bind chitotriose less efficiently than native lysozyme (by a factor of 10) but to possess only 4% of the specific activity of lysozyme toward hydrolysis of chitotriose and 10-12% toward hydrolysis of *M. lysodeikticus*. Rechromatography of this preparation resulted in its separation from contaminating lysozyme with the result that its specific activity, using *M. lysodeikticus* as substrate, was found to be, in the best preparations, 0-0.5% that of native enzyme. Because of the nature of the substrate it was difficult to be more quantitative than this. In addition, assay of activity using chitotriose as a substrate was complicated by the conditions used, 40° for 1 hr at pH 5.5. It was not possible to eliminate the possibility of limited ester hydrolysis. However, we feel that this derivative is essentially devoid of enzymatic activity. Therefore, it seems reasonable to suggest that the integrity of a single carboxyl residue is essential for lysozyme catalysis. This result is in agreement with the suggestions of Blake et al. (1967a) based on model building of saccharide molecules which were fitted into the cleft in the surface of their lysozyme model, as extensions of the known lysozyme-chitotriose nonproductive complex. It is also in agreement with the pH profile for lysozyme catalysis of various synthetic substrates (Raftery and Rand-Meir, 1968). It would seem therefore that a specific carboxyl side chain in lysozyme is critical for catalysis by the enzyme although it is not possible from the present studies to specify the exact role played by this group.
The next chapter in this thesis describes the identification of this particular residue as regards its position in the amino acid sequence of the enzyme.
References


Dahlquist, F. W., and Raftery, M. A. (1968), Biochemistry 7, 3269, 3277.


Park, J. T., and Johnson, M. J. (1949), J. Biol. Chem. 181, 149.


Table 1. Ethyl Ester Content of Ethanol-HCl Esterified Lysozyme

<table>
<thead>
<tr>
<th>Method of analysis</th>
<th>Moles ester per mole enzyme</th>
</tr>
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<tr>
<td>1. Ethoxyl content(^a)</td>
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</tr>
<tr>
<td>2. Hydroxamate(^b)</td>
<td>4.9</td>
</tr>
<tr>
<td>3. Glycine amide incorporation(^c)</td>
<td>5.3</td>
</tr>
</tbody>
</table>

\(^a\) This analysis was performed by the Microanalytical Laboratory, University of California, Berkeley, California.

\(^b\) Performed according to Yasphe et al. (1960).

\(^c\) Performed by the method of Hoare and Koshland, 1966, 1967 using 5 M guanidine hydrochloride as solvent for the coupling reaction on the performic acid oxidized protein.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theory</th>
<th>Control</th>
<th>Per-ester</th>
<th>[Et₂O][BF₄]⁻</th>
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<th>Component III</th>
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</table>
Table 2. (Cont’d)

a Values are expressed in residues per molecule.
b All molar ratios were calculated relative to a value of 12.00 for alanine.
c This material was obtained by esterification of lysozyme in ethanol-HCl.
d This material had been reacted with 1.0 M triethylloxonium fluoroborate at pH 4.5 and was shown to contain an average of 5.5 ester groups per molecule of enzyme by base uptake.
e This material was isolated chromatographically and was shown to be essentially devoid of enzymatic activity. It contained one ethyl ester per molecule.
f This material was also isolated chromatographically and was shown to contain one labile ester. See text for description of its isolation and properties.
Table 3. Estimation of Free Carboxyl Groups in Lysozyme and in Derivative II by Coupling with Glycine Amide\textsuperscript{a, b}

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Theory</th>
<th>Control lysozyme</th>
<th>Lysozyme coupled</th>
<th>Derivative II coupled</th>
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</table>

\textsuperscript{a} The method used was that of Hoare and Koshland (1966, 1967) as described in the experimental section.

\textsuperscript{b} Values are expressed as residues per molecule.

\textsuperscript{c} All molar ratios were calculated by normalizing to a value of 12.00 for alanine.
Figure 1. Plot of the inverse of the chemical shift of the acetamido methyl resonance of 2-acetamido-2-deoxy-D-glucopyranose (at mutarotational equilibrium) in association with the ethyl ester derivative of lysozyme vs. the total concentration of 2-acetamido-2-deoxy-D-glucopyranose. Measurements were made in 0.1 M citrate buffer (pH 5.5) at 40° and 60 M Hz, lysozyme concentration 2.8 × 10^{-3} M.
Figure 2. The pathway for the reaction of triethyloxonium fluoroborate with carboxyl groups.
Figure 3. A plot of the per cent activity remaining after successive treatments of lysozyme with 0.2 M oxonium salt. The ethylations were carried out at pH 4.5 at a lysozyme concentration of $8.5 \times 10^{-4}$ M.
REAGENT TO ENZYME MOLAR RATIO, x10^{-2}

ACTIVITY, %

0 2 4 6 8 10 12
Figure 4. Chromatography of ethylated derivatives of lysozyme on Bio-Rex 70 cation-exchange resin.
(A) Native lysozyme. (B) Lysozyme treated with 0.1 M triethylxonium fluoroborate at pH 4.0. Component I is the unreacted lysozyme. Component II was not obtained in sufficient quantity to identify. Component III is a mono-ethyl ester of lysozyme. (C) Component III incubated at pH 7.2 for 48 hr at room temperature.
LYSOZYME REACTED AT pH 4

LYSOZYME

REVERTED

A

B

C

Absorbance at 575 nm

Fraction number
Figure 5. Chromatography on Bio-Rex 70 of ethylated derivatives of lysozyme formed at pH 4.5. (A) Lysozyme with 0.2 M triethylloxonium fluoroborate. Component IV is a diethyl ester containing the esters of components II and III. (B) The above mixture was incubated at 7.2 for 20 hr at room temperature.
Figure 6. Chromatography with gradient system on Bio-Rex 70 of $^{14}$C-labeled derivatives of lysozyme formed at pH 4.5 with about 0.15 M oxonium salt.
Figure 7. A plot of the rechromatography of component II (-•-) and the enzymatic activity of each fraction (-o-).
CHAPTER III
Identification of a Critical Carboxyl in Lysozyme

Introduction

Recent discussions of hen egg-white lysozyme generally have attributed its glycosidic activity to the side chains of Glu-35 and Asp-52. There is some kinetic evidence that carboxyls participate (Osawa, 1966; Raftery and Rand-Meir, 1968; Rupley, 1967). Also, modification of all "available" carboxyls inactivates the enzyme (Fraenkel-Conrat, 1950; Hoare and Koshland, 1967; Parsons et al., 1969). However, the evidence that Glu-35 and Asp-52 are the specific carboxyls which are involved has been of a presumptive nature based on the elegant results of Phillips, Blake, and co-workers for the crystalline enzyme and on their model building (Blake et al., 1967a and b; Blake, 1966; Blake et al., 1965).

In the previous chapter we reported the utilization of triethyl-oxonium fluoroborate to achieve mild selective esterification of carboxylates in lysozyme. We were able to isolate and partially characterize two single ethyl ester derivatives of the enzyme. One of these is enzymatically inactive but still retains the capability of binding the competitive inhibitor chitotriose. Figure 1 illustrates the chromatographic pattern obtained in the separation of a mixture of the lysozyme ester derivatives. Component II is the enzymatically
inactive single ester discussed here. This chapter describes the identification of this important carboxylic acid side chain.
Experimental

Materials. -- Hen egg-white lysozyme (lot 77B-8040) was purchased from Sigma Chemical Co. Bovine $\alpha$-chymotrypsin (lot T-97207) was obtained from Armour Research Division, aminopeptidase M (lot 51132, 12,500 mEU/mg) from Rohm and Haas, subtilisin Carlsberg (lot 50624) from Novo Industries Copenhagen, and carboxypeptidase A (lot 762) from Worthington Biochemical Corp. Ninhydrin was a product of the Pierce Chemical Co. and aspartic acid was obtained from Eastman Kodak. Poly-$\ell$-glutamic acid was a product of Schwarz Bioresearch, Inc. Standardization buffers for the pH meter were from Beckman.

The $\beta$-ethyl ester of aspartic acid was synthesized by refluxing a mixture of 5.0 g $d\ell$-Asp·HCl and 1.1 g anhydrous HCl in 50 ml absolute ethanol for 15 min. The resulting warm solution was brought to cloudiness with dry ether and set aside (Bergmann and Zervas, 1933). Crystalline $\beta$-$d\ell$-ethyl aspartic acid hydrochloride (2.8 g, mp 174.5-177.5°C) was obtained. The structure was confirmed by nuclear magnetic resonance spectroscopy.

The $\gamma$-ethyl ester of glutamic acid was quickly synthesized in micro-amount by esterification of 1 mg of poly-$\ell$-glutamic acid with triethylloxonium fluoroborate in a manner similar to the lysozyme esterification. The esterified polymer was treated with subtilisin and aminopeptidase M as described below, yielding a solution containing essentially only $\ell$-glutamic acid and $\gamma$-ethyl-$\ell$-glutamate in about
1:3 ratio.

Analytical Methods. -- General procedures. Acid hydrolysis of peptides was effected in constant-boiling HCl under vacuum for 20 hours at 105°C. Base hydrolysis of protein samples was effected in 4 N barium hydroxide by the method of Noltman (1962). Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer. The sodium buffer system usually employed was similar to that of Spackman (1963). Asparagine and β-ethyl aspartic acid (β-EtAsp) were completely resolved from the other amino acids at 25°C in a 0.3 M lithium (citrate) buffer pH 2.80, described in technical bulletin A-TB-044 from Spinco. Only the first buffer, eluting amino acids through glycine, was used. The values of amino acids normally found in acid hydrolysates were calibrated by standard mixtures (Beckman Co., Spinco Division). Other amino acids (β-EtAsp, Asn, Gln, Try) were calibrated with weighed amounts of the carefully dried amino acids.

Visible and ultraviolet absorbancies were determined with a Gilford Model 240 spectrophotometer. Scintillation counting of 14C derivatives was performed in 15 ml of Bray's solution (Bray, 1960) in a Packard Model 527 scintillation spectrometer. Nuclear magnetic resonance spectra were obtained on a Varian Model A60 spectrometer. A Radiometer PHM26 with a combination calomel-glass electrode was used to obtain pH readings.

Stability of the inactive ester. Two mg of the 14C-labeled inactive ester (see below for its isolation), also referred to as
component II, which contained 1600 cpm was performic acid oxidized at -10°C by the method of Hirs (1956). The product was taken up in 1.8 ml water and a 0.8 ml aliquot counted for radioactive content. To the rest, 0.1 ml of 0.2 M phosphate pH 7.2 was added, the pH was adjusted to 7.3, and 0.1 mg of trypsin was added. After nine hours at room temperature the protein was lyophilized and then taken up in 1.1 ml H₂O. A 0.8 ml aliquot was counted. The original ester (0.6 mg) in 0.8 ml of water also was counted.

**Total enzymatic digestion.** A solution of 0.3 mg subtilisin in 1.2 ml of 0.1 M phosphate pH 7.0 was added to 5 mg each of oxidized native lysozyme and inactive ester and stirred at room temperature. After 3 hours the oxidized protein had completely dissolved. After 4 hours more, 2.0 mg of solid aminopeptidase M, which cleaves all peptide bonds (Pfleiderer et al., 1963), was added. After 20 hours of further digestion the solution was frozen. Aliquots were analyzed for amino acid composition in both the sodium and the lithium buffer systems.

An aliquot (0.20 ml) of each digest was treated with two drops of 1 N LiOH in 0.50 ml of water (to dilute the cation concentrations), giving a solution of pH 11. This was incubated at 40°C for 30 minutes after which one drop of 6 N HCl was added and the solution analyzed in the lithium buffer system.

**α-Chymotryptic digestion.** Five mg of oxidized native lysozyme or inactive ester was dissolved in 1.5 ml of 10⁻³ M phosphate and 10⁻³ M CaCl₂, pH 7.0. The acidic protein lowered the pH of the
solution to about 4 where it is soluble. The digestion was carried out in a 12 × 50 mm test tube fitted with magnetic stirring, a combination pH electrode, and a micrometer mounted 1 ml syringe with No. 22 U-bent delivery needle. A solution of 0.3 mg α-chymotrypsin in 0.3 ml of the same buffer was prepared in a separate syringe. The pH of the digest solution was rapidly raised to 7.0, precipitating the oxidized protein, and the α-chymotrypsin quickly injected. The pH was rapidly brought up to 6.98 ± 0.03 and maintained at that pH with 0.1 N NaOH. The mixture cleared in 10 minutes. After 4 hours at 25°C, the base in the syringe was changed to 1 N HCl and the pH of the peptide solution lowered to 3.10. This solution was applied immediately to the peptide column.

Peptide chromatography. Chromatography of peptide solutions was performed on columns (0.9 × 17 cm) maintained at 35.0°C of a spherical, nominally 7.5% cross-linked, sulfonated polystyrene resin from Spinco, Type PA-35 (Hill and Delaney, 1967). The eluting buffer gradient was formed by means of a nine chamber Autograd and was similar to one suggested by D. I. Schmidt (1966). Three stock buffers were prepared of (1) 0.2 N sodium formate adjusted to pH 3.20 with HCl, (2) 2.0 N sodium acetate of about pH 6.8. Table 1 gives the initial compositions of the nine chambers. A flow rate of 50 ml/hr, which resulted in a pressure of 300 psi, was maintained by means of a Milton Roy Model 196 positive displacement pump. The entire effluent was fed into the amino acid analyzer. The analyzer ninhydrin system was operated in the usual manner and the resulting
peptide map was recorded at slow chart speed (Hill and Delaney, 1967). Thirteen hours were required for the chromatography. The resin was regenerated in the column with 0.2 N NaOH (30 min) followed by the starting pH 3.20 buffer (1 hr) with no increase in back pressure.

**Preparative Methods.** -- $^{14}$C Labeled inactive ester. A mixture of lysozyme ethyl esters obtained by reaction with $^{14}$C labeled triethylloxonium fluoroborate had been prepared and analyzed previously (Parsons et al., 1969). After the addition of 50 mg each of nonradioactive component II and component IV to this mixture as carriers, $^{14}$C labeled component II was isolated by the method previously described (Figure 1). The other components appearing in Figure 1 were isolated at the same time also, but will not be discussed further here.

$\alpha$-Chymotryptic digestion and peptide chromatography. The preparative digestion was similar to the analytical one, except that 25 mg of oxidized lysozyme or inactive ester was dissolved in 7.0 ml dilute phosphate buffer contained in a 10 ml beaker and 1.5 mg $\alpha$-chymotrypsin in 0.5 ml of the same buffer was added. Chromatography of the resulting peptides was performed as before. The column effluent was collected in 5 ml fractions, but only in the region of interest indicated by the analytical results spanning the 4th to the 7th hours of chromatography. The peptide regions were located by manual ninhydrin analysis on a 250 $\mu$ aliquot of each fraction. The four fractions containing the peptide of interest or the region into which (for lysozyme) or from which (for the ester) the peptide moved
were pooled.

Removal of sodium ions and peptide contaminants due to peak
tailing. A pooled fraction was evaporated to 1 ml on a rotary
evaporator under vacuum at 35°C. The viscous solution was trans­
ferred with washing to a 10 ml graduated cylinder, made up to 2.1 ml,
and mixed with 0.7 ml of isopropanol. This solution was chromato­
graphed on a column (2.5 x 40 cm) of superfine Sephadex G-25
(defined) equilibrated in 25% isopropanol-water (v/v) which was 0.036
M with respect to ammonium formate and adjusted to pH 4.6 with
formic acid. Five ml fractions were collected. The peptide peaks
were located by ultraviolet absorptivity at 230 m\(\mu\). Each UV absorb­
ing fraction was pooled, the isopropanol was evaporated under
vacuum at 35°C and the resulting 25 ml of water solution lyophilized.
The residue was taken up in 1.00 ml water and frozen to give a stock
solution of peptide. Amino acid analyses were performed both with
and without acid hydrolysis on 0.10 ml portions of these stock
solutions.

Carboxypeptidase A digestion of peptide C-15. Carboxypeptidase
A (4 mg) was solublized by the method of Fraenkel-Conrat et al.
(1955) and made up to give a solution of 0.72 mg/ml carboxypeptidase
A (CPA) in 0.2 M N-ethylmorpholine (acetate) at pH 7.6. This was
treated with diisopropyl fluorophosphate (Potts, 1967). About 200
m\(\mu\) moles of peptide C-15 (0.20 ml and 0.40 ml of the stock solutions
from lysozyme and the ester, respectively) were made up to 1.00 ml
with 0.10 ml of the CPA solution and 0.26 M or 0.36 M morpholine
buffer to give similar digest solutions containing 0.20 M N-ethylmorpholine (acetate) at pH 7.5. A blank lacking C-15 peptide was prepared also. These were incubated at 25°C and 200 λ aliquots periodically were removed, pipetted into 0.5 ml of 0.2 M citrate pH 2.2 and quick frozen until amino acid analysis. The 25 minute sample from the ester digest was adjusted to pH 11 with NaOH, incubated at 35°C for 1 hour, and then brought to pH 2.5 with HCl.
Results

The elution pattern obtained from the separation of the $^{14}$C-labeled oxonium salt esterified derivatives appears in Figure 1. About 30 mg of the labeled inactive protein (Parsons et al., 1969) (750 cpm/mg), component II, was obtained from fractions 133 to 154. When this labeled ester was oxidized in performic acid, 90% of the counts were recovered after lyophilization, indicating that the ester was stable to this treatment. Incubation of oxidized labeled component II at pH 7.2 for 9 hours at room temperature showed that 90% of the counts surviving oxidation were recovered after lyophilization, again indicating the stability of the ester group to this treatment.

Basic hydrolysis of the inactive protein indicated that its tryptophane content was identical with that of lysozyme. Acid hydrolysis previously had shown the same results for the other amino acids (Parsons et al., 1969). The total enzymatic hydrolysis gave values for the amino acid composition in reasonable agreement with the accepted composition (Canfield, 1963a; Jollès et al., 1963), except for low values of aspartic acid, glycine, and arginine (see Tables 2 and 3). The incomplete yield of aspartic acid always occurred and has been noted in other cases of total enzymatic digestion (Hill and Schmidt, 1962). It is apparent from Tables 2 and 3 that the ester derivative gave the same ratios of amino acids as native lysozyme except for one less Asp. One additional peak after Glu and partly resolved from Pro was observed in the amino acid
analyzer trace of the ester digest run in the usual sodium system. This position corresponded to that of authentic $\beta$-ethyl aspartic acid. No other new significant peaks occurred which were not present in the lysozyme digest. No peak at the position of $\gamma$-ethyl glutamic acid, between valine and isoleucine, was observed. Since the proline peak has a low 570 m$\mu$ color intensity, the $\beta$-EtAsp peak could be integrated even though it was only half resolved. The presence of $\beta$-EtAsp in the ester digest was further shown by the identity of the new peak with authentic $\beta$-EtAsp in a lithium buffer system, where it again occurred between Glu (plus Gln) and Pro but this time was completely resolved. The $\beta$-EtAsp peak is very distinctive since it possesses a greater color intensity in the 440 m$\mu$ channel than in the alternate 570 m$\mu$ channel. The 570 m$\mu$ color intensity given by $\beta$-EtAsp was reproducibly about 1/3 that of most other amino acids. Treatment of the ester digest with dilute base resulted in the disappearance of the peak attributed to $\beta$-EtAsp and in an increase equal to one residue of the aspartic acid peak (Table 3).

In the proteolytic digestion with $\alpha$-chymotrypsin most of the base uptake was complete after two hours. When the resulting peptide solution was chromatographed 18 peaks could be identified. The two analytical peptide maps obtained for lysozyme and the inactive ester were essentially identical except for a single major peak (Figure 2). The peak occurring at 5$\frac{1}{2}$ hours in the chromatogram of the lysozyme digest had moved back to 6 hours in the ester pattern. A small peak due to ester-hydrolyzed peptide or a different peptide also falling at
51\frac{1}{2} hours remained in the position formerly occupied by the ester peptide. This provided evidence that the shift in position was significant.

When the lysozyme peptide occurring at 51\frac{1}{2} hrs, fractions 23-26 in Figure 3A, was isolated preparatively and chromatographed on Sephadex, three UV absorbing peaks appeared which were preceded by an ill-defined forerun (see Figure 4A). The forerun was probably composed of contaminants due to peak tailing associated with peptide columns. Peaks 2 and 3 contained no amino acids after acid hydrolysis. Peak 3 was due to the concentrated sodium salts. Peak 1 gave the amino acid analysis after acid hydrolysis shown in Table 4. Without acid hydrolysis no amino acids were detected. The analysis corresponded well to peptide C-15 of Canfield's work which has the structure Arg-45, Asn-46, Thr-47, Asp-48, Gly-49, Ser-50, Thr-51, Asp-52, Tyr-53 (Canfield and Anfinsen, 1963; Canfield and Liu, 1965). Two aspartic acid residues, at positions 48 and 52, occur in this sequence. The yield of peptide was 1000 m\mu moles (60%).

Work-up in an identical manner of the region in the lysozyme pattern occurring at 6 hours, fractions 30-33 in Figure 3A, gave the Sephadex pattern shown in Figure 4A'. It was evident that no peptides of low ninhydrin reactivity occurred in this region.

When the new peptide from the inactive ester, fractions 30 to 33 in Figure 3B, was isolated preparatively, the Sephadex pattern shown in Figure 4B resulted. Amino acid analysis of the acid hydrolyzed peptide revealed that it too was C-15 (Table 4).
The yield was 500 mµ moles (30%). The yield, which is based on the weight of material oxidized, is low because of salt in the original isolated protein. No free amino acids occurred with the isolated peptide. Fractions 24-27 of Figure 3B gave the Sephadex pattern of Figure 4B'. This result indicated that little of the ester had hydrolyzed and that no peptide of low ninhydrin reactivity was present at $5\frac{1}{2}$ hours along with C-15.

Carboxypeptidase A digestion of C-15 from lysozyme gave an immediate quantitative release of tyrosine-53 and a very slow release of aspartic-52 (Figure 5A), as expected (Ambler, 1967). No other amino acids were observed. The digestion of peptide C-15 from the ester gave quite different results. Tyrosine-53 was again quickly released. No aspartic acid was released but instead β-ethyl aspartic acid was released at a moderate rate (Figure 5B). The increased rate of release of an uncharged β derivative of aspartic acid is expected (Ambler, 1967). Furthermore, a small amount of threonine-51 was released. No other amino acids were observed in significant amounts. When the 25 minute sample from the ester peptide was treated with dilute base before amino acid analysis, 32.4 mµ moles of aspartic acid was found. This datum, plotted as mµ moles β-ethyl aspartic acid, fits into the curve of Figure 5B well. This is over ten times the amount of aspartic acid released from the native lysozyme peptide after 25 minutes.
Discussion

Before discussing the procedure which identified the inactive ester, we should mention briefly some early unsuccessful procedures. In order to avoid the possibility of ester hydrolysis, attempts were made to convert the inactive lysozyme derivative to a hydrazide or to a hydroxamic acid. Such ester conversions normally occur readily, can be made specific and have been utilized previously on protein esters with success (e.g., Blumenfeld and Gallop, 1962; Gallop et al., 1959). However, with component II, none of the expected product could be detected although model esters and the component III ester underwent the expected conversions. LiBH₄ reduction (Wilcox, 1967) was tried also without success. Even after vigorous treatment, resulting in the appearance of one half residue of homoserine from native lysozyme, no additional homoserine was found from component II. Perhaps related to these properties is the fact that incubation of the inactive ester at pH 10 did not regenerate enzymatic activity, while incubation at pH 2 did (Parsons et al., 1969). It is interesting to note that each of the above reactions which failed depends upon an initial nucleophilic attack on the ester while the one reaction which succeeded depends upon an initial protonation of the ester.

Component II was previously shown to be an ester by indirect means. The coupling of glycine amide to the oxidized protein by means of a carbodiimide (Hoare and Koshland, 1967) demonstrated the disappearance of one carboxyl group. Because component II
failed to undergo typical ester reactions, further confirmation of its identity was desirable. The first step was to rule out the modification of the other amino acids. Acid hydrolysis combined with basic hydrolysis completed the elimination of all the amino acids except Asp, Glu, Asn, Gln, and the C-terminal Leu as to having been ethylated by the oxonium salt. Assuming that the presence of an ester would be confirmed, the carbodiimide result rules out Asn and Gln. Also, the C-terminal sequence is known to be not critical (Jollès, 1967). After the radioactive label was shown to be stable to performic acid oxidation (also expected from the carbodiimide results) we sought to observe the ester directly. This was done by total enzymatic hydrolysis. The identification of one residue of β-ethyl aspartic acid in the component II total enzymatic hydrolysate was quite certain by virtue of three identical characteristics with authentic β-EtAsp in two different amino acid analyzer buffer systems. Because of its low specific radioactivity, the 14C-labeled derivative was not utilized to locate the site of esterification. The α-chymotryptic peptide map readily showed that Asp-48 or Asp-52, occurring in C-15, was the site of esterification. The C-15 peptide is ideally suited for investigation of Asp-52 with carboxypeptidase A since the C-terminal Tyr-53 residue would be expected to separate rapidly and quantitatively, giving rise to an "internal standard", and to give no complications in the release of the second residue.

Carboxypeptidase A digestion showed much more rapid release of β-EtAsp from the component II peptide than of Asp from the native
lysozyme peptide. Because β-EtAsp-52 was observed directly, it was felt unnecessary to examine Asp-48 for modification since a negative result for an ester, which is hydrolyzable, would be inconclusive. The site of esterification in component II was therefore shown to be at aspartic acid 52.

It was previously shown that this derivative is enzymatically inactive while retaining the capability of binding the inhibitor chitotriose. Thus, the above identification constitutes direct chemical evidence that the catalytic functionality of hen egg-white lysozyme occurs in the region of Asp-52. This result is in agreement with the hydrolytic mechanism suggested by Phillips and Blake (Blake, 1966; Blake et al., 1967a). It is not, by itself, certain proof that Asp-52 and Glu-35 are indeed the participating groups. In a study complementary to this one, Lin and Koshland (1969) prepared an active lysozyme derivative in which all of the carboxyl groups except Asp-52 and Glu-35 were modified to an extent greater than the loss of enzymatic activity. The two studies together strongly indicate that Asp-52 and Glu-35 are in fact the important carboxyls.
References


Blumenfeld, O. O. and Gallop, P. M. (1962), Biochemistry, 950.


Pfleiderer, G. and Celliers, P. G. (1963), Biochem. Z. 339, 186.


Spackman, D. H. (1963), Federation Proc. 22, 244.

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<th>Chamber</th>
<th>Buffer No. 1 pH 3.20 (ml)</th>
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<th>Buffer No. 3 pH 6.8 (ml)</th>
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Table 2. Amino Acid Compositions of Total Enzymatic Digests Determined in Sodium Buffer System

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<tr>
<th>Amino acid</th>
<th>Amino acid ratios&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Inactive ester</th>
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<td>4.7</td>
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<sup>a</sup> Residues per lysozyme molecule.
Table 3. Amino Acid Compositions of Total Enzymatic Digests Determined in Lithium Buffer System

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<th>Inactive ester</th>
<th>Lysozyme after pH 11</th>
<th>Inactive ester after pH 11</th>
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*Residues per lysozyme molecule.
Table 4. Amino Acid Compositions of $5\frac{1}{2}$ Hour and 6 Hour α Chymotryptic Peptides (Sodium Buffer)

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<th>Inactive ester 6 hr peptide</th>
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<td>543 (1)</td>
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Figure 1. Preparative chromatography of $^{14}$C-labeled lysozyme esters on Bio-Rex 70 (2.5 × 40 cm), 7°C, pH 7.19, salt gradient from 0.16 M $\text{PO}_4$ (Na$^+$) (3 chambers of 500 ml) to 0.20 M $\text{PO}_4$ (Na$^+$) containing 0.50 M KCl (1 chamber of 500 ml), 8 ml fractions. Component I is native lysozyme; component II is an enzymatically inactive single ester derivative.
Figure 2. Analytical $\alpha$-chymotryptic peptide map of performic acid oxidized proteins. Chromatography on Beckman-Spinco resin PA-35 (0.9 × 17 cm), 35°C, nine chamber gradient from 0.2 N sodium formate pH 3.20 to 2 N sodium acetate pH 6.8, amino acid analyzer ninhydrin system.

A. Lysozyme

B. Inactive ester

A single peptide peak in B moved from $5\frac{1}{2}$ hrs to 6 hrs elution time.
Figure 3. Preparative $\alpha$-chymotryptic peptide chromatogram from the 4th to the 7th hours. Same chromatographic conditions as in Figure 2, 5 ml fractions, manual ninhydrin analyses.

A. Lysozyme

B. Inactive ester

Fractions enclosed by bars were pooled.
Figure 4. Sephadex G-25 chromatography of pooled regions from Figure 3. Chromatography in 25% isopropanol-0.036 M ammonium formate pH 4.6, 5 ml fractions, 230 m\(\mu\) absorptivity of each fraction read.

A. Lysozyme C-15, fractions 23-26 of Figure 3A.
A'. Lysozyme, fractions 30-33 of Figure 3A.

B. Inactive ester C-15, fractions 30-33 of Figure 3B.
B'. Inactive ester, fractions 24-27 of Figure 3B.

Fractions enclosed by bars were pooled and analyzed for amino acid content.
Figure 5. Release of amino acids from C-15 peptide by carboxypeptidase A in N-ethylmorpholine acetate pH 7.5 at 25°C, versus time.

A. Lysozyme

B. Inactive ester

Ester sample taken at 25 minutes (-o-) was treated with base before amino acid analysis. Other samples (-•-) were chromatographed directly.
CHAPTER IV

Difference pH Titrations of the Catalytic Carboxyls of Lysozyme in Various Ionic Strength Solutions

Introduction

There have been few accurate measurements of the properties of individual carboxyl groups in proteins. Measurements which have been made are usually based on apparent pK's as manifested in enzyme kinetic and binding studies or poorly delineated spectral changes. Confirmation that the observed effect originates from a carboxyl group has been sought by determining the apparent heat of ionization (Dixon and Webb, 1964) or the effect of solvent perturbation on the ionization (Findlay et al., 1962). This approach can mis-identify the type of ionizable group since it generally assumes that the group exhibits ordinary small molecule properties modified but slightly by the protein. Most carboxyls in enzymes are quite ordinary, but the extraordinary carboxyls are the most interesting and probably the most functional. Furthermore, beyond the mere assignment of an apparent pK to a type of group is the far greater problem of assignment to a particular residue in the amino acid sequence. The above methods are inferential in this regard.

A more reliable approach to the characterization of individual carboxyl groups in enzymes would involve prominent labeling of a known important residue. The degree of ionization under sets of
equilibrium conditions would be observed directly, and the results of these independent studies could be correlated with pH effects on the enzyme-substrate interactions. Ideally, a label such as carbon-13, which would not disturb the system, should be used. This is not now generally possible. An alternative is to change the chemical structure of the carboxyl; for example, by esterification. Since the group then no longer titrates the modified enzyme must be compared to the native in order to characterize the functional carboxyl.

An obvious comparison to make when dealing with a singly esterified enzyme is a difference pH titration. It is worth noting that this is a preferred comparison even if another such as a pH-dependent perturbed protein UV absorbance is available. This is so because the hydrogen ion titration of a group in a well characterized protein is a measurement which has a predictable span. Accordingly, more complex difference curves can be fitted with confidence. The availability of the β-ethyl ester of aspartic acid residue 52 (Parsons and Raftery, 1969) presents us with the opportunity to determine the titration behavior of the lysozyme active site. This is of clear interest in understanding the detailed physical structure of this region and the catalytic mechanism.

Information about the properties of glutamic acid residue 35 (Glu 35) has been obtained previously. A single carboxyl of pK 6.0 to 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 area of the enzyme consistent with all of these effects.

In contrast to Glu 35, no information has been available about Asp 52. Kinetic data suggest that its pK is between 3.5 and 4.5 in the catalytic complex (Parsons et al., 1969; Rupley, 1967; 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 chapter that these effects do not arise from Asp 52. However, other unassigned observations which will be discussed below probably originate from this residue.

This chapter reports on some aspects of the pH titration of the Asp 52 ester derivative. The results are presented in the form of a difference titration from which four micro-constants for the ionization of Asp 52 and Glu 35 in the native enzyme have been accurately
determined in solutions containing 0.02 to 0.50 molar KCl. The next chapter reports on temperature effects.
Experimental *

Lysozyme was obtained from Sigma Chemical Co. (lot 28B-8120). Native enzyme was prepared chromatographically pure directly from the commercial lysozyme. The Asp 52 ester derivative was prepared from 10 g of lysozyme and isolated chromatographically as previously described (Parsons et al., 1969). Both preparations were dialyzed separately for two days at 4°C 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 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 enzyme.

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. Solutions for the titration in about 0.02 M KCl were prepared in 0.01 M KCl. The UV difference spectrum (320 to 260 mµ) of the two solutions was obtained on a Cary Model 14 spectrophotometer utilizing a 0.1 OD slide wire and matched UV cells of 0.5 mm path length (Research and Industrial Instruments Division of Beckman, Fullerton, California, Model UV 10). The two protein concentrations were adjusted until the

*Abbreviations used are: UV, ultraviolet; OD, optical density; µ, ionic strength.*
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 is 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 2026C), 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 are 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°C. Nitrogen was saturated with water vapor at the same temperature and the line leading to the titration vessel thermostated. The pH meter was 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 removal of CO₂. The response of the electrodes was checked occasionally between titrations 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 2 ml pipette. For titrations at salt concentrations above 0.15 M the appropriate weight of crystalline KCl was added. The solution was equilibrated one hour 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.

A preliminary titration of lysozyme vs. lysozyme plus KOAc was performed. The effect of ionic strength was observed at 0.02, 0.15, 0.22, 0.30, and 0.50 molar KCl. Because 0.150 N titrant was used for all these, the ionic strength varied somewhat throughout titrations at \( \mu \) values other than 0.15 M, and the stated KCl concentrations varied by about ±0.007 molar. The enzymatic activity of the Asp 52 ester preparation was checked after the titration.

All calculations were carried out on an Olivetti Underwood Programma 101 desk-top computer. Two sigma errors are reported for the slopes of Figure 9.
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 mμ and about pH 6 mean 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 547.0 small Agla units (0.1094 ml) for native lysozyme and 489.5 units for the derivative. The difference in water volumes between the two solutions towards the end of the titration requires 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. Identical results
are obtained whether one titrates from high to low pH or vice versa. Also, titrations at 15 mg/ml and 5 mg/ml protein yield the same difference data. All the titrations reported here were performed from high to low pH at 15 mg/ml protein.

Providing that the esterification of Asp 52 did not perturb the pK's of any other groups in lysozyme which titrate between pH 3.0 and 9.0, one would expect to obtain a difference titration curve for Asp 52 similar to that shown in Figure 1. The data in Figure 2 show that this is not the case. Instead, there apparently are three ionizations reflected in the data. The two rising sections of the curve (read left to right) between pH's 3.0 to 4.8 and 5.7 to 9.0 indicate that native lysozyme was titrating more protons than the derivative was over these pH intervals. The section from pH 4.8 to pH 5.7 indicates that the derivative was titrating an equal number of protons over this pH interval.

Figure 2 is readily explained as resulting from the perturbation of a group of about pK 6 in native lysozyme to a lower value in the ester derivative. The perturbed group is certainly Glu 35 since it is located quite close to Asp 52 in the tertiary structure. No other group is as close. Absence of the negative electric field from Asp 52 at pH 5-6 in the ester derivative would lower the pK of Glu 35. There is probably only one other group in lysozyme with a pK in this range. His 15 has a pK of 5.8 (Bradbury and Wilairat, 1967), but it is on the other side of the molecule. Also, a downward perturbation of a group with a high pK is indicated by the chromatographic behavior of the
Asp 52 ester derivative (Chapter II). This derivative was not held up on ion exchange chromatography on a carboxylate resin as much as other single ester derivatives relative to native lysozyme at pH 7.2 (Parsons et al., 1969). 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 Glu 35 in the native enzyme predominates between pH 5.7 and 9.0. Other interpretations of Figure 2 are possible but extremely unlikely in view of all the evidence cited immediately above and in the introduction to this chapter. This result is quite reasonable since there are no other titratable groups in close proximity to Asp 52 and Glu 35 in the three-dimensional structure of lysozyme.

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 mµ 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 63 ± 1 small Agla units. Utilizing this theoretical span to the difference titration, the points were fitted with the sum of two positive and one negative ideal titration curves. As will be discussed below, the two positive curves for Asp 52 and Glu 35 in the native enzyme determine "titration" constants G1 and G2, respectively. The pG values are nearly, but not quite, the correct values for the
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 ideal curve on the experimental points gave a close fit. Figure 2 was best fitted by $pG_1$ equal to 4.40 and $pG_2$ equal to 6.10 in native lysozyme, and $pK_2$ equal to 5.20 in the derivative. A repeat titration 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.

The data from titrations in KCl concentrations varying from 0.02 M to 0.50 M are shown in Figures 3, 4, 5, and 6. The curves in Figures 3 and 6 were obtained from identical samples with crystalline KCl added to the latter titration 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 Figure 6. All difference results were fitted as described above.
Discussion

Interpretation and Assignment of the Difference pH Titration Curve. -- The average number of protons dissociated from a lysozyme molecule can be written as

\[ \bar{h} = \sum_i \alpha_i \]  

(1)

where \( \alpha \) is the probability that the \( i \)th group will be dissociated; \( \alpha_i \) is some complicated function of the pH, the dissociation constant describing the pH at which the \( i \)th group is one-half ionized, and the variable electrostatic interactions between the \( i \)th group and other charged groups (Edsall and Wyman, 1958). The dissociation constant of each group is not generally the intrinsic constant for that type of group. It may have been raised or lowered by electrostatic, hydrogen bonding, or hydrophobic effects.

Esterification of one carboxyl generally will affect \( \alpha \) for distal titratable groups only slightly, and the titrations of distal groups will cancel closely in a difference pH titration. Only those groups which are significantly perturbed by the esterification will appear in \( \Delta \bar{h} \). If we assume that one group interacts strongly with the esterified carboxyl and is the only other significantly perturbed group we can write Equation 2 for the difference pH result,

\[ \Delta \bar{h} = \frac{G_1 e^{-\phi(Z)}}{H^+ + G_1 e^{-\phi(Z)}} + \frac{G_2 e^{-\phi(Z)}}{H^+ + G_2 e^{-\phi(Z)}} - \frac{k_2 e^{-\phi(Z)}}{H^+ + k_2 e^{-\phi(Z)}} \]  

(2)
$G_1$ 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 $\phi(Z)$ is some complicated function of the variable charge interactions with the rest of the protein (Tanford, 1961). Although progress has been made (Orttung, 1968, 1969) exact calculation of the local electrostatic potential at the $i$th group is presently most difficult and somewhat uncertain. As an approximation to $\phi$ we could perhaps utilize the Linderstrøm-Lang model for "generalized" electrostatic interactions in proteins (Tanford, 1962). In any case, as discussed next, it fortuitously occurs that in lysozyme we usually can ignore $\phi$ to a first approximation.

Lysozyme binds chloride ions (Carr, 1953) such that its electrokinetic potential changes little below pH 6 (Beychok and Warner, 1959). Furthermore, only two protons titrate between pH 6 to 9 and one of them is Glu 35 itself (Sakakibara and Hamaguchi, 1968). Thus the total charge on lysozyme changes very little below pH 9. One could therefore hope that the relatively weak interactions with distal charged groups would average to a nearly constant electrostatic potential in the region of the active site. In solutions of sufficient ionic strength where electrostatic interactions are decreased we can take the exponential terms in Equation (2) as equal to one to a close approximation.
Thus the analysis suggested by Figures 2 through 6 and expressed by Equation (2) is that of a dibasic acid, where a strong interaction between Asp 52 and Glu 35 is accounted for and these two groups are subject to a "generalized" electrostatic potential from the rest of the lysozyme molecule. In the presence of sufficient salt this potential will be relatively constant so that the shape of the difference pH titration curve will be nearly ideal. The ionic strength will affect the magnitude of the potential and thus the dissociation constants of Asp 52 and Glu 35. The very close fit of the data in Figures 2, 4, 5, and 6 to Equation (2) with $\phi$ taken as zero, under the restriction of an independently determined difference span, lends strong support to the adequacy of this scheme. Figure 3 is also in fact good confirmation of the analysis. In a low ionic strength solution where the $\phi$ term is probably significant (and also where the salt concentration was changing significantly throughout the titration) the data can be approximately fitted with only three titration constants. Even in a low salt solution no other group titratable between pH 3 and 9 interacts strongly with Asp 52 or Glu 35. It should be noted that there are no apparent complications from the reported dimerization of lysozyme (Sophianopoulos and van Holde, 1964).

In fitting the titration curve of a dibasic acid one obtains the two "titration" constants, $G_1$ and $G_2$, for the curve. These constants are related to the two molecular dissociation constants, $K_1$ and $K_2$, describing the first and second states in the titration, by Equations (3a) and (3b),
The two molecular dissociation constants are dependent upon four micro-constants for the individual groups according to Equations (4a) and (4b),

\[ K_1 = G_1 + G_2 \quad (3a) \]
\[ K_1 K_2 = G_1 G_2. \quad (3b) \]

The two molecular dissociation constants are dependent upon four micro-constants for the individual groups according to Equations (4a) and (4b),

\[ k_1 + k_2 = K_1 \quad (4a) \]
\[ \frac{1}{k_3} + \frac{1}{k_4} = \frac{1}{K_2}. \quad (4b) \]

A complete discussion of these relationships may be found in Edsall and Wyman (1958) and in Simms (1926).

The two ionization pathways with their four micro-constants are illustrated in Figure 7 for the interacting Asp 52-Glu 35 system. \( H_a \) represents a proton bound to the Asp 52 side chain and \( H_g \), to the Glu 35 side chain. Thus step \( k_1 \) is Asp 52 ionizing with Glu 35 protonated, step \( k_2 \) is Glu 35 ionizing with Asp 52 protonated, step \( k_3 \) is Glu 35 ionizing with Asp 52 already ionized and step \( k_4 \) is Asp 52 ionizing with Glu 35 already ionized. 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 micro-constants can be calculated from the titration constants by utilizing Equations
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 7. 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 $p_{k_2}$ more toward the value in the presence of ionized Asp 52. Thus the calculation of all four micro-constants on the basis of the value for Glu 35' provides 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 1. It should be noted that the Asp 52 and Glu 35 ionizations overlap only moderately since $p_{k_1}$ and $p_{k_3}$ differ from $p_{G_1}$ and $p_{G_2}$, 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 through 6 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 $p_{k_1}$ and $p_{k_3}$ to be ±0.1 pH unit. The values of $p_{k_2}$ and $p_{k_4}$ are more uncertain. Thus the micro-constants in Figure 7 for Asp 52 and Glu 35 at 25°C in 0.15 M KCl are $p_{k_1}$, 4.43; $p_{k_2}$, 5.22; $p_{k_3}$, 6.01; and $p_{k_4}$, 5.22.
The Shape of the Asp 52 Ionization Curve. -- We can calculate the shape of the Asp 52 ionization curve by utilizing the micro-constants and Equation (5),

$$\text{Fraction Asp 52 \over \text{Ionized}} = \frac{k_1[(H^+) + k_3]}{k_1[(H^+) + k_3][(H^+) + k_2]}.$$ \hspace{1cm} (5)

As shown in Figure 8 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°C in 0.15 M KCl. If the actual curve were approximated by an ideal one as in Figure 8, a pK of 4.6 would be estimated. A similar shaped curve is obtained for Glu 35 from an equation analogous to Equation (5).

Ionic Strength Effects on the Micro-Constants. -- Table 1 demonstrates that a change in the ionic strength of the solution has a large effect on several of the micro-constants. 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). It should be noted that 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). At low ionic strength the pk's are lower, indicating an increased positive electrostatic potential from the rest of the protein in the region of the active site. Those micro-constants for the ionizations which occur with the other catalytic group protonated, pk_1 and pk_2, are lowered
the most. The ionization of Glu 35 when Asp 52 is already ionized, 
$p_{k_3}$, is nearly independent of the ionic strength, thus indicating that 
the electrostatic potentials from ionized Asp 52 and the rest of the 
protein essentially cancel in the region of Glu 35.

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 approximation to estimate the consequences. Because they are buried within an enzyme of net positive 
electrostatic potential, Asp 52 and Glu 35 can not be approached closely by cations in solution. We can treat their ionizations as 
occurring within the volume of a impenetrable sphere which approxi­mates size of the lysozyme molecule with the enzyme charge spread evenly over the surface. It should be noted that 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. Because we are calculating the effects of a generalized potential, the 
estimates should be carried out only for the two microscopic ioniza­tions which occur when the other catalytic group is protonated. We 
can write Equation (6) to describe the approximate dependency of 
micro-constants $k_1$ and $k_2$ on ionic strength (Edsall and Wyman, 1958).

$$pk = pk^0 - \frac{Z^2 \varepsilon^2}{4.6 DkT} \left( \frac{1}{b} - \frac{K}{1 + \kappa a} \right) \quad (6a)$$

$$\kappa = \left( \frac{4\pi \varepsilon^2}{DkT} \mu \right)^{\frac{1}{2}}. \quad (6b)$$
Z is the number of charges on the rest of the protein, \( \varepsilon \) is the protonic charge, \( D \) is the dielectric constant of the surrounding medium, \( k \) is the Boltzmann constant, \( T \) is the absolute temperature, \( b \) 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 \( (a = b + r_s) \), \( \mu \) is the ionic strength, \( p_k \) is the negative logarithm of the observed micro-constant, and \( p_k^0 \) is the value which the ionization would exhibit if the electrostatic potential from the rest of the protein were not present.

A plot of \( p_k \) vs. \( \kappa/(1 + \kappa a) \) should be a straight line with slope equal to \( Z^2 \varepsilon^2/4.6 DkT \). The intercept at \( \kappa/(1 + \kappa a) \) equal to zero, where the ionic strength is zero, is \( p_k^0 \)-slope/b. When the ionic strength is infinite \( \kappa/(1 + \kappa a) \) equals 1/a and \( p_k = p_k^0 \)-slope \((1/b - 1/a)\).

The relevant calculations of \( \kappa \) are listed in Table 2. To calculate \( \kappa/(1 + \kappa a) \) we have taken \( b \) equal to 19Å, 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 21Å, a number based on the usual 2Å radius for the chloride ion (Edsall and Wyman, 1958). The corresponding values of \( \kappa/(1 + \kappa a) \) are also listed in Table 2. These are plotted vs. \( p_k \) and \( p_k^2 \) in Figure 9 where it can be seen that a straight line fits the points quite well.

The least-squares slopes and two sigma errors in Figure 9 are \((5.63 \pm .46) \times 10^{-7} \) cm for \( p_k \) and \((3.91 \pm .62) \times 10^{-7} \) cm for \( p_k^2 \). From the slopes we can calculate that \( Z \) equals 6 for Asp 52 and 5 for Glu 35.
That is, Asp 52 sees about 6 net positive charges and Glu 35, about 5, from the rest of the lysozyme molecule. Extrapolation to the intercepts corresponding to either $\mu$ equal to zero or $\mu$ equal to infinity yields the same value of $p_{k^0}$; for $p_{k_1^0}$ the value is 5.4 and for $p_{k_2^0}$ it is 5.9. A reasonable error in these numbers is ±0.2 pH units.

**Effective Dielectric Constants.** Bjerrum (1923) was the first to attempt to explain the influence of a nearby charged group on the ionization constant of an acid. Kirkwood and Westheimer (1935) modified the earlier theory by taking into account the difference in dielectric constant between the internal volume of the ionizing molecule and the solvent. The equation takes on a simple form if one utilizes an effective dielectric constant, $D_e$. The theory was originally proposed in order to calculate the distance between charged groups in molecules, but since we already know the distance between Asp 52 and Glu 35 from the crystallographic structure, we can calculate the effective dielectric constant from Equation (7).

$$D_e = \frac{\epsilon^2}{2.3 \ kT \ r \ \Delta p_k}.$$  

(7)

Here $r$ is the distance between the center of the carboxyls, which we will take to be halfway between the oxygens of each carboxyl, $\Delta p_k$ is the change in $p_k$ caused by the presence of the other ionized group, and the rest of the symbols have been defined previously.
The lysozyme coordinates, kindly supplied by D. C. Phillips, indicate that Asp 52 and Glu 35 are 7.13 Å apart. The difference between pk₂ and pk₃ (or pk₁ and pk₄) gives the electrostatic interaction as equal to 0.8 log units. From this number we calculate that the effective dielectric constant between Asp 52 and Glu 35 is 43, compared to 79 for water. A detailed interpretation of \( D_e \) will have to await extensive theoretical calculations.

Another calculation given by Equation (10) can be made to estimate the effective dielectric constants immediately surrounding Glu 35 and Asp 52 (Tanford, 1961).

\[
\frac{1}{D_e} = \frac{4.6 \alpha kT \Delta \phi}{\varepsilon^2} + \frac{1}{D_w}.
\]

Here \( \alpha \) is the radius of the carboxylate ion, \( \Delta \phi \) is the change in \( \phi \) due to the transfer of the carboxylate from water to a solvent of effective dielectric constant \( D_e \), \( D_w \) is the dielectric constant of water, and the other symbols have been defined previously. Taking \( \alpha \) as 2 Å and \( \Delta \phi \) as equal to \( \phi^0 - 4.6 \) (the \( \phi \) expected in water for a glutamic or aspartic side chain) we calculate that \( D_e \) equals 30 for Glu 35 and 37 for Asp 52. These are approximate and not subject to detailed interpretation.
Conclusion

The two catalytic carboxyls of lysozyme clearly can be regarded in their charge interactions as analogous to a moderately interacting dibasic acid. They are not perturbed significantly by any other particular group titrating between pH 3 and 9, but they are subject to a relatively constant, averaged electrostatic potential from the rest of the enzyme. Furthermore, the conformation of the active site as evidenced by the pk's of the two carboxyls appears to be stable to wide ranges of ionic strength.

Although the ionization constant of Asp 52 would appear normal on the basis of its observed pK, for example, from Figure 2 or 8, in fact it is not normal. Figure 9 demonstrates that Asp 52 is in a slightly "hydrophobic" environment which gives it an "intrinsic" pk 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'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. The negative charge on ionized Asp 52 raises the ionization constant of Glu 35 to 6.01. The final result, then, is that an "intrinsic" ionization advantage of only about 0.5 pH unit on the part of Asp 52 has been increased to 1.4 pH units (pK 4.5-pK 5.9, Figure 8) by electrostatic interactions.
The validity and approximate 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 NMR techniques Dahlquist and Raftery (1968) had 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 8 which would result from an observation which was directly dependent on the ionization of Asp 52 as determined here. Also, the good linearity of the Debye-Hückel plot supports the validity of Equations (2) and (6). The total charges on the lysozyme molecule at pH's 4.4 and 5.2 determined from the slopes of Figure 9 are in approximate agreement with the values determined by Beychok and Warner (1959) from electrophoretic mobilities. Thus the interpretation of the difference pH titrations is internally consistent and quantitatively supported by existing relevant data.
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Sakakibara, R., and Hamaguchi, K. (1968), J. Biochem. 64, 613.


Table 1. Ionic Strength Dependence of the Micro-Constants for the Asp 52 and Glu 35 Ionizations

<table>
<thead>
<tr>
<th>Ionic strength, M</th>
<th>0.02</th>
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<th>0.22</th>
<th>0.30</th>
<th>0.50</th>
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<td>pk₁ (Asp 52)</td>
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<td>4.40</td>
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<td>4.56</td>
<td>4.69</td>
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<td></td>
<td></td>
<td>4.43</td>
<td></td>
<td></td>
<td>4.77</td>
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<td>pk₂ (Glu 35')</td>
<td>~4.8</td>
<td>5.25</td>
<td>5.30</td>
<td>5.30</td>
<td>5.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.20</td>
<td></td>
<td></td>
<td>5.40</td>
</tr>
<tr>
<td>pk₃ (Glu 35)</td>
<td>~5.9</td>
<td>6.00</td>
<td>6.04</td>
<td>5.94</td>
<td>6.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.03</td>
<td></td>
<td></td>
<td>5.93</td>
</tr>
<tr>
<td>pk₄ (Asp 52)</td>
<td>~4.9</td>
<td>5.15</td>
<td>5.30</td>
<td>5.20</td>
<td>5.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.29</td>
<td></td>
<td></td>
<td>5.30</td>
</tr>
</tbody>
</table>

a At 25°C in KCl as calculated by Equations (3) and (4) applied to Figures 2, 3, 4, 5, and 6, as well as two figures not shown, by assuming k₂.
Table 2. Debye-Hückel Parameters

<table>
<thead>
<tr>
<th>Ionic strength, molar</th>
<th>$\kappa \times 10^{-7}$†</th>
<th>$\frac{\kappa}{1 + \kappa a} \times 10^{-7}$*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>0.02</td>
<td>0.463</td>
<td>0.234</td>
</tr>
<tr>
<td>0.15</td>
<td>1.272</td>
<td>0.343</td>
</tr>
<tr>
<td>0.22</td>
<td>1.540</td>
<td>0.364</td>
</tr>
<tr>
<td>0.30</td>
<td>1.800</td>
<td>0.376</td>
</tr>
<tr>
<td>0.50</td>
<td>2.32</td>
<td>0.395</td>
</tr>
<tr>
<td>$\infty$</td>
<td>$\infty$</td>
<td>0.476</td>
</tr>
</tbody>
</table>

† At 25°C.

* a equal to 21Å.
Figure 1. The difference titration of $2 \times 10^{-4}$ M acetic acid in $3.5 \times 10^{-4}$ M lysozyme, at $25^\circ$C in 0.15 M KCl. The determined pK of 4.50 is the value expected under these conditions.
Figure 2. Difference titration of 2.00 ml of lysozyme vs. the Asp 52 ester derivative, both 15 mg/ml (about $1 \times 10^{-3}$ M), in 0.150 M KCl at 25.0°C. The total uptake of 0.150 N HCl from pH 9.000 to pH 3.000 was 547.0 small Agla units (0.1094 ml) for lysozyme and 489.5 small units (0.0979 ml) for the derivative. The expected difference due to one amino acid side chain ester is 63 small Agla units which has been normalized to a span of one. The data points were fitted with the sum of two positive and one negative ideal titration curves. 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 titration in approximately 0.02 M KCl at 25.0°C. The data cannot be fitted well with only three titration constants because of the increased importance of generalized electrostatic interactions.
Figure 4. The difference titration in approximately 0.22 M KCl at 25.0°C.
0.22 M KCl

\[
\begin{align*}
pG_1 & = 4.50 \\
pG_2 & = 6.10 \\
\text{Glu} 35' \ & pk_2 \ 5.30
\end{align*}
\]
Figure 5. The difference titration in approximately 0.30 M KCl at 25.0°C.
0.30M KCl

Difference, Moles H⁺/Mole Asp 52 Ester

pG₁  4.50
pG₂  6.00
-Glu 35'  pk₂  5.30

pH 3.0  4.0  5.0  6.0  7.0  8.0  9.0
Figure 6. The difference titration in approximately 0.50 M KCl at 25.0°C. This curve was obtained from the same solutions used in Figure 3 but with crystalline KCl added to attain 0.50 M KCl. A separate titration under similar conditions gave titration constants of 4.70, 6.00, and 5.40.
0.50 M KCl

Difference, Moles H⁺ / Mole Asp 52 Ester

pG₁ 4.65
pG₂ 6.10
-Glu 35', pk₂ 5.55

pH
Figure 7. 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 micro-constants $k_1$ and $k_3$ with $^{\Theta}\text{LH}_g$ the catalytically active species.
Figure 8. The ionization of Asp 52 in native lysozyme. The heavy curve was calculated from Equation (5) using the average of the micro-constants determined at 25°C 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 Glu 35 ionization curve would be of similar shape with the point of half ionization occurring at pH 5.9.
Figure 9. The Debye-Hückel theory (Eq. (6)) applied to the effect of the lysozyme charge on the ionizations of Asp 52 and Glu 35, each ionizing when the other is protonated, at 25°C in varying concentrations of KCl. The ionic strengths corresponding to each $\kappa/(1 + \kappa a)$ are listed in Table 2. The value of $a$ has been taken as 21Å and $b$, as 19Å. The values which $p k_1$ and $p k_2$ would have if the rest of the lysozyme molecule were not charged are $p k_1$ equal to 5.4 and $p k_2$ equal to 5.9.
CHAPTER V

Thermodynamics of Ionization of the Catalytic Carboxyls of Lysozyme

Introduction

It was shown in Chapter IV that both Asp 52 and Glu 35 are in environments which may be grossly classified as slightly "hydrophobic". This label is of little value in describing the interactions between the two residues and their respective neighbors in the protein tertiary structure. The thermodynamics of ionization for the groups would help in this respect. With the aid of the X-ray map of the active site we could hope to interpret these thermodynamic parameters successfully.

It has previously been shown that the $\Delta H$ of ionization for a group of $pK$ 6.2, which can now be assigned to Glu 35, was about $3 \pm 1$ kcal/mole (Donovan et al., 1961). A group observed by Kowalski and Shimmel (1969) which we believe to be Asp 52 (Chapter IV), also had a significant positive heat of ionization.

The difference titration between the aspartic acid residue 52 derivative and native lysozyme has been performed at several temperatures in order to obtain heats and entropies of ionization for the micro-constants of the Asp 52 and Glu 35 ionizations. An attempt also has been made to approximately correct the observed thermodynamics for the influence of the charge on the protein.
Experimental

Aliquots of the same preparations of the derivative and of native lysozyme used for the difference titration at 25.0° presented in Figure 2 of Chapter IV were used here. Titrations were performed at 1.6° and 40.0° in the manner previously described. Difference data were fitted as before with the sum of two positive and one negative titration curves. From these titration constants the four micro-constants (Chapter IV, Figure 7) were calculated by assuming $k_2$. 
Results

Difference Curves. -- The two difference curves obtained from identical solutions at 1.6° and 40° are shown in Figure 1. The curve obtained at 25° is shown in Figure 2 of Chapter IV. There is a large shift toward acid pH in the difference titration at high temperature. The significant deviation at 1.6° C below pH 4 from the three parameter curve is attributed to experimental error because of the longer time required for equilibration at low temperature.

Observed Heats and Entropies of Ionization for the Micro-Constants. -- The micro-constants calculated from Equations (3) and (4) of Chapter IV for Asp 52 and Glu 35 at 1.6°, 25° and 40° are listed in Table 1. Van't Hoff plots of the micro-constants can be made in order to calculate apparent heats of ionization. The micro-constants determined here are not strictly thermodynamic constants but rather apparent constants, thus yielding apparent heats and entropies of ionization.

The plots for pk₁, pk₂, and pk₃ are shown in Figure 2. The average observed heats of ionization and their two sigma errors over the temperature range 1.6 to 40°C in 0.15 M KCl are listed in Table 2 and are 3.5±1.4 kcal/mole for k₁, 1.9±1.2 kcal/mole for k₂, 2.9±0.8 kcal/mole for k₃, and 4.5±3.2 kcal/mole for k₄. The apparent entropies of ionization calculated from ΔS = (ΔHobs - 2.3 RT pk)/T at 25°C are also listed and are -9±5 e.u. for k₁, -18±4 e.u. for k₂, -18±2 e.u. for k₃, and -9±11 e.u. for k₄.
Discussion

**Observed Heats and Entropies of Ionization.** -- Because of the large changes in heat capacity associated with carboxyl ionizations the van't Hoff plot is not strictly appropriate. However, by plotting data above and below 25°C, a ΔH close to the correct value at 25°C will be obtained from the van't Hoff slope. Caution should be exercised before accepting the plot. A significant change in the charge on the protein at the same pH at different temperatures would lead to a shift in pk's even though the actual heats of ionization were zero. Because the overall titration of lysozyme below pH 7 is only slightly dependent on temperature (Tanford and Wagner, 1954) and much of the dependency is accounted for by Glu 35 itself, the problem is not encountered here. It should be noted that the thermal denaturation temperature for lysozyme is well above 40°C at all titrated pH values (Sophianopoulos and Weiss, 1964).

**Heats and Entropies of Ionization in Uncharged Lysozyme.** -- The values of pk_1^0 and pk_2^0 in Chapter IV, for which all charge interactions have been approximately corrected, are more indicative of the "solvent" environments of Asp 52 and Glu 35 than any of the other pk's are. Thus it was determined that Asp 52 is in a somewhat "hydrophobic" environment even though its observed titration behavior is approximately normal. It would be informative if we similarly could correct the observed heats and entropies of ionization of k_1 and k_2 for the influence of the protein charge. The observed
thermodynamic constants at 25 °C in 0.15 M KCl, listed in Table 2, can be divided into two contributions, an "intrinsic" part and an electrostatic part, as in Equations (1a, b, and c).

\[
\Delta F_{\text{obs}} = \Delta F^0 + \Delta F(\text{el}) \tag{1a}
\]
\[
\Delta S_{\text{obs}} = \Delta S^0 + \Delta S(\text{el}) \tag{1b}
\]
\[
\Delta H_{\text{obs}} = \Delta H^0 + \Delta H(\text{el}). \tag{1c}
\]

The electrostatic free energy of ionization due to the rest of the protein, \(\Delta F(\text{el})\), readily is obtained from the difference between the \(pk\) in 0.15 M KCl and \(pk^0\). These values are listed in Table 3 for \(k_1\) and \(k_2\).

If one converts \((pk^0 - pk)\) in Equation (6a) of Chapter IV to free energy, differentiates this expression with respect to temperature and takes \(\Delta H\) as constant with respect to temperature, Equation (2) can be obtained for the electrostatic entropy.

\[ -\frac{d \Delta F(\text{el})}{dT} = \Delta S(\text{el}) = \Delta F(\text{el}) \frac{d \ln D}{dT}. \tag{2} \]

The term in Equation (6a) of Chapter IV involving \(\kappa\) is quite negligible in the derivative and has been dropped. Westheimer and Kirkwood (1946) have discussed the accuracy of Equation (2). It must be regarded as quite crude, exhibiting errors of up to a factor of two compared to experimental values measured for small molecules.
However, the equation at least gives the correct sign for the electrostatic contribution in Equation (1b). The derivative of $\ln D$ with respect to temperature is given by Akerlöf (1932) as $-0.0047^\circ\text{K}^{-1}$. Since $\Delta F(\text{el})$ is negative also, the contribution to the observed entropy change because of the charge on lysozyme is positive. This is readily conceptualized as follows. When an uncharged carboxylic acid ionizes, the charge on the carboxylate molecule increases by one unit with a consequent increase in hydration and decrease in entropy. When Asp 52 or Glu 35 ionizes, the total charge on the lysozyme molecule decreases. Thus, relative to a simple carboxyl group, there is less loss of entropy when Asp 52 or Glu 35 ionizes and the observed $\Delta S$ of ionization is more positive than an equivalent ionization in uncharged lysozyme.

Using the Akerlöf value in Equation (2) we calculate that $\Delta S(\text{el})$ equals $+6.5$ e.u. for $k_1$ and $+4.5$ e.u. for $k_2$. Therefore $\Delta H(\text{el})$ equals $+0.5$ kcal/mole for $k_1$ and $+0.4$ kcal/mole for $k_2$. Using these values in Equations (1b) and (1c) we calculate that $\Delta S^0$ equals $-15$ e.u. for $k_1$ and $-23$ e.u. for $k_2$ and that $\Delta H^0$ equals $+3$ kcal/mole for $k_1$ and $+1.5$ kcal/mole for $k_2$. The corrected ionization parameters for Asp 52 and Glu 35 are listed in Table 3. They must be regarded as approximate.

**Aspartic Acid Residue 52.** -- The observed heat and entropy of ionization for $k_1$ in Table 2 are both substantially different from the values usually found in proteins. Typical values at $25^\circ$ are $-1.5$ to $+1.5$ kcal/mole for $\Delta H$ and $-18$ to $-21$ e.u. for $\Delta S$ (Tanford, 1962).
Since $k_1$ dominates the overall ionization of Asp 52, the thermo-
dynamic values for $k_1$ are nearly those which would be deduced from
any direct measurement on Asp 52.

These values do not arise from two enzyme states of
significantly different structure, with the enthalpy and entropy change
spread over a large part of the cleft. Physical evidence, including
optical rotation and viscosity (Jirgensons, 1958; Yang and Foster,
1955), inhibitor binding studies utilizing nuclear magnetic resonance
(Dahlquist and Raftery, 1968), and the integrity of the Asp 52 ester
derivative as evidenced by retained inhibitor binding (Parsons et al.,
1969), the small UV difference spectrum with native enzyme and the
titration data, all indicates that there is no measurable enzyme
conformational transition coupled to the Asp 52 ionization.

The values for $\Delta H_{\text{obs}}$ and $\Delta S_{\text{obs}}$ are somewhat misleading if
one were to attempt an interpretation of them. Thus one might
attribute special structural significance to the rather small negative
entropy of ionization of -9 e.u. However, this observed value
becomes more normal when corrected for the lysozyme charge.

The corrected $\Delta H^0$ and $\Delta S^0$ in Table 3 for $k_1$ still both appear
to be abnormal, although the uncertainties in these numbers do not
allow us to conclude this definitively. It is interesting, though, that
these "solvent" determined parameters may be readily rationalized
in terms of the possible interactions between Asp 52 and the
surrounding protein structure as deduced by X-ray crystallography.
The Asp 52 carboxyl is in a position where it could accept hydrogen bonds from Asn 46 and Asn 59. Blake et al. (1967) suggested that the formation of these bonds to the ionized form of Asp 52 would be consistent with a lowering of its pK and therefore with its catalytic role. Formation of these intramolecular hydrogen bonds between side chains oriented by hydrophobic interactions would displace hydrogen bonded water and lead to an increase in the entropy of the system. A good discussion of the thermodynamics of hydrogen bonding in proteins is given by Nemethy et al. (1963). The somewhat small corrected decrease in entropy resulting from the ionization of Asp 52, -15 e.u., is consistent with the X-ray structural deduction. Partial solvation of Asp 52 by the surrounding protein is not as effective as full hydration would be, as evidenced by the large positive heat of ionization of 3 kcal/mole, but the built-in entropy advantage nearly compensates for this.

Some chemical properties of the Asp 52 β-ethyl ester derivative also indicate that this carboxyl does not interact strongly with water. The ester group of the native derivative is inert to attack by hydroxylamine, hydrazine, and hydroxide ion, thus demonstrating rather tenacious steric hindrance around Asp 52 (Parsons and Raftery, 1969). On the other hand, the ester does hydrolyze slowly at pH 2 and room temperature (Parsons et al., 1969), conditions not far removed from those where lysozyme undergoes a reversible thermal denaturation (Sophianopoulos and Weiss, 1964).
Thus the chemical reactivity, the thermodynamics of ionization, the surrounding effective dielectric constant and the surrounding tertiary structure of Asp 52 all indicate that this part of the active site does not interact strongly with water and also has little freedom of motion. This does not mean that a subtle reorganization of the cleft could not occur during binding or catalysis but only that the bottom of the cleft is not a loose structure.

A catalytic region configurationally stabilized by hydrogen bonding to Asp 52 has several consequences relevant to the proposed stabilized carbonium ion mechanism of lysozyme (Dahlquist et al., 1968; Blake et al., 1967; Rand-Meir et al., 1969). In pairing the carbonium ion with Asp 52, lysozyme apparently prevents collapse of the insipient ion pair to a covalent transition state in the rate determining step. Hydrogen bonding to ionized Asp 52 would deactive it as a nucleophile; a rigid environment around Asp 52 sterically could prevent collapse.

Also, for a carboxyl group which must be maintained in the anionic state in a substrate complex at acidic pH, less hydration is a quite desirable situation. If Asp 52 were strongly hydrated, then tightly bound water molecules would have to be displaced when substrate bound, and that at least would slow the binding association rate. Furthermore, if in the uncomplexed state Asp 52 depended upon hydration to maintain its low pK, it is unlikely that the bound saccharide could supply equal solvation. The result would be both an increased pK for Asp 52 when complexed and a decreased association.
constant for substrate when Asp 52 was ionized. Both effects are counter-catalytic. From the same initial situation, binding could induce a conformational change which solvated Asp 52 with the protein and substrate and maintained the same pK, but this would cost binding energy. Thus built-in solvation of a buried catalytic group would seem to have evolutionary advantage. In Chapter VI of this Thesis we show that bound inhibitors and substrates in fact do not change the pK of Asp 52. It seems very unlikely that a significant dehydration step would be balanced out nearly exactly by quite different solvation modes in the enzyme-substrate complex.

Glutamic Acid Residue 35. -- We will discuss Glu 35 only briefly since it has been characterized previously by others. The heats of ionization for Glu 35 listed in Table 2 are abnormally positive, but the entropies of ionization are within the normal range. Since they essentially dominate the Glu 35 ionization behavior, the parameters listed in Table 2 for \( k_3 \) are those which would be deduced from a direct observation of Glu 35. Table 3 gives an estimate for these values in "neutralized" lysozyme. The entropy is then less negative than normal but not by very much. The crystallographic structure of lysozyme shows Glu 35 to be in a hydrophobic region of the cleft. It is not surprising that a decreased hydration is reflected by a positive \( \Delta H \) of ionization. An increased electrostatic work of charging Glu 35 makes \( \Delta H \) even more positive (Table 2) in the presence of ionized Asp 52. The normal value of \( \Delta S^0 \) indicates that either water in the cleft or part of the protein structure, or both, is oriented
specifically by the Glu 35 ionization.
Conclusion

Our models in Chapters IV and V for analyzing the ionizations of Asp 52 and Glu 35 have been the simplest possible. Their apparent adequacy is indeed somewhat surprising. However, these titration data are the first of their type to our knowledge which are so accurate and so well defined as to origin. Thus the work in one sense is exploring the applicability of these simple models to protein titrations of this type. As has been described previously, lysozyme is probably an ideal protein in which to attempt such an extensive analysis. Our deductions about the nature of the Asp 52 and Glu 35 ionizations, and the actual adequacy of our models, await other confirmatory or modificatory data.
References


Dahlquist, F. W., and Raftery, M. A. (1968), Biochemistry 7, 3277.


Jirgensons, B. (1958), Arch. Biochem. Biophys. 74, 70.


Nemethy, G., Steinberg, I. Z., and Scheraga, H. A. (1963), Biopolymers 1, 43.


Table 1. Temperature Dependence of the Micro-Constants for the Asp 52 and Glu 35 Ionizations

<table>
<thead>
<tr>
<th>Temperature</th>
<th>1.6°C</th>
<th>25°C</th>
<th>40°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pk₁ (Asp 52)</td>
<td>4.69</td>
<td>4.40</td>
<td>4.36</td>
</tr>
<tr>
<td>pk₂ (Glu 35')</td>
<td>5.30</td>
<td>5.25</td>
<td>5.10</td>
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<tr>
<td>pk₃ (Glu 35)</td>
<td>6.21</td>
<td>6.00</td>
<td>5.93</td>
</tr>
<tr>
<td>pk₄ (Asp 52)</td>
<td>5.60</td>
<td>5.15</td>
<td>5.19</td>
</tr>
</tbody>
</table>

*In 0.15 M KCl, calculated by assuming k₂ in Equations (3) and (4) of Chapter IV as applied to Figure 1 of this chapter and Figure 1 of Chapter IV as well as one other figure for 25°C which is not shown.*
Table 2. Observed Thermodynamics of Ionization for Asp 52 and Glu 35$^a$

<table>
<thead>
<tr>
<th>Ionization step$^b$</th>
<th>pk$^c$</th>
<th>$\Delta H_{obs}^d$ (kcal/mole)</th>
<th>$\Delta S_{obs}^e$ (cal/deg-mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$ (Asp 52)</td>
<td>4.43</td>
<td>$3.5 \pm 1.4$</td>
<td>$-9 \pm 5$</td>
</tr>
<tr>
<td>$k_2$ (Glu 35')</td>
<td>5.22</td>
<td>$1.9 \pm 1.2$</td>
<td>$-18 \pm 4$</td>
</tr>
<tr>
<td>$k_3$ (Glu 35)</td>
<td>6.01</td>
<td>$2.9 \pm 0.6$</td>
<td>$-18 \pm 2$</td>
</tr>
<tr>
<td>$k_4$ (Asp 52)</td>
<td>5.22</td>
<td>$4.5 \pm 3.2$</td>
<td>$-9 \pm 11$</td>
</tr>
</tbody>
</table>

$^a$ Determined from Figure 2.

$^b$ Micro-constants identified in Figure 7 of Chapter IV.

$^c$ At 25°C in 0.15 M KCl.

$^d$ Observed apparent heat of ionization with two sigma error.

$^e$ Observed apparent entropy of ionization with two sigma error.
Table 3. Thermodynamics of Ionization for Asp 52 and Glu 35 in Uncharged Lysozyme

<table>
<thead>
<tr>
<th>Ionization step</th>
<th>pk(^a)</th>
<th>pk(^b)</th>
<th>ΔF(el)(^c) kcal/mole</th>
<th>ΔS(el)(^d) e. u.</th>
<th>ΔH(^0)(^e) kcal/mole</th>
<th>ΔS(^0)(^f) e. u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>k(_1) (Asp 52)</td>
<td>4.43</td>
<td>5.4</td>
<td>-1.4</td>
<td>6.5</td>
<td>3</td>
<td>-15</td>
</tr>
<tr>
<td>k(<em>2) (Glu 35(</em>\prime))</td>
<td>5.22</td>
<td>5.9</td>
<td>-0.96</td>
<td>4.5</td>
<td>1.5</td>
<td>-23</td>
</tr>
</tbody>
</table>

\(^a\) Measured at 25°C in 0.15 M KCl.

\(^b\) Obtained from Figure 9 of Chapter IV.

\(^c\) The electrostatic free energy difference corresponding to pk-pk\(^0\).

\(^d\) The electrostatic entropy of ionization calculated from ΔF(el) d ln D/dT.

\(^e\) The corrected heat of ionization that k would exhibit if lysozyme were not charged.

\(^f\) The corrected entropy of ionization at 25°C that k would exhibit if lysozyme were not charged.
Figure 1. Difference titrations of lysozyme vs. the Asp 52 ester derivative at 1.6°C and 40°C. These titrations were performed on aliquots of the same solutions used for Figure 2 in Chapter IV.
Figure 2. Van't Hoff plots of the data from Table 1. The determined heats of ionization are listed in Table 2 along with their two sigma errors.
CHAPTER VI

Some pK Determinations for the Cleft Carboxyls in Lysozyme-Inhibitor Complexes as Determined by Difference pH Titrations

Introduction

The successful measurement of some properties of Asp-52 and Glu-35 in the free enzyme 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-βCH₃* and NAG₃ 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

*Abbreviations used in this chapter are: NAG-βCH₃, methyl-2-acetamido-2-deoxy-β-D-glucopyranoside; NAG₂-βCH₃, methyl-β-chitobioside; NAG₃, chitotriose; NAG₃-Glu-βCH₃, methyl-β(1-4)-chitotriosyl-β-D-glucopyranoside; NAG-Glu-βφNO₃, nitrophenyl-2-acetamido-2-deoxy-D-glucopyranosyl-β(1-4)-D-glucopyranoside.
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 chapter reports on difference titrations carried out in the presence of methyl-\(\beta\)-NAG, methyl-\(\beta\)-chitobioside, chitotriose, methyl-\(\beta\)(1-4)-chitotriosyl-\(\beta\)-D-glucopyranoside, and glycol chitin. The curves reflect the behavior of both catalytic carboxyls as well as Asp-101 under conditions when various binding subsites are occupied.
Experimental

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 Company) by gel-filtration on Bio Gel P-2 (Raftery et al., 1969). All the above products were treated for several minutes 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. Five grams chitotetraose were incubated 4 hr with 50 g methyl-β-glucoside (Sigma) and 0.5 g lysozyme in 1 liter of 0.1 M acetate pH 5.5 at 37°C. The solution was acidified to pH 3, ultrafiltered to remove the enzyme (UM-1 membrane, Amicon Corp.), and stirred one 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. One hundred ml fractions were collected and the chitosyl glucosides located by their absorbance at 220 nm. The fractions corresponding to methyl-β(1-4)-chitotriosyl-β-D-glucopyranoside were pooled and lyophilized. The structure proof for similar transferase products
has been described previously (Rand-Meir et al., 1969). 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 same preparations of lysozyme and of the Asp-52 ester that were used for the titrations in Chapters IV and V were used here.

**Methods.** -- The preparation of matched protein solutions and the titration procedure are in general the same as in Chapter IV. 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, one 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°C 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 one-half hour. 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 in Chapter IV for reasons discussed in the Results Section.

The pH dependency for the dissociation constant of the trimer-Asp-52 ester derivative complex was determined by a UV difference spectrum method (Dahlquist et al., 1966) at 22°C in 0.01 M citrate-phosphate mixed buffers made up to 0.15 M potassium ion with KCl.
Results

The 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°C in 0.15 M KCl. We first will mention 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 (Chapter IV, Figure 2). 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. This is not due to electrostatic effects since a similar titration in 0.5 M KCl suffered the same ill-fit (Figure 5).

From the results of Chapter IV, we have no reason to believe that the electrostatic potential changes significantly in the region of the active site over this pH range.

Some difficulties are expected in these titrations because we have 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 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, 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 to 5.0 might be explained.

The pH Dependence of the Association of NAG₃. -- 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 pKₛ through the pH range 3.5 to 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 pKₛ on pH over the pH range 3 to 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. The perturbation due to Asp-101 must have a positive titration curve of about pK 3.6 and a negative curve of about pK 4.2. Figure 6 also tells us that the Glu-35
pk₂ 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 nearly unchanged from the value 4.4 determined in the free enzyme and the Glu-35 titration constant will be about 6.5. With these five initial guesses for the three positive and two negative titration curves that are required 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 pG₁, 6.50 for pG₂, 3.70 for the pK of Asp-101, 4.10 for the pK of Asp-101' in the derivative, and 5.10 for pk₂ of Glu-35' in the derivative. The titration constants G₁, G₂, and k₂ have the same meaning and relationships to Asp-52 and Glu-35 as in Chapter IV. The other titration constants are taken as the true apparent dissociation constants for those groups.

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 the derivative in each of these Figures was taken to be variable between 3.6 and 4.2 with the value for Asp-101 more certain at about 4.2. In general, the data between pH 4 to 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 positively charged 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 apparently contains a titratable group which is not removed by MB-1 resin. The titration of this group occurred between pH 6 to 8 and consumed a volume of acid equal to half that 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 $H^+$ per mole Asp-52 ester and reasonably can be identified as due to Glu-35. Its approximate pK is 8.0 to 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 probably 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 will be saturated by chitotriose under these conditions (Figure 6). No information is available on the binding of the other inhibitors and 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.*, 1968; Rand-Meir *et al.*, 1969; Rupley, 1967). Little hydrolysis will have occurred under the conditions of these titrations. Significant hydrolysis of glycol chitin will occur (Hamaguchi *et al.*, 1960).
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. Also, the occurrence of transglycosylation will help to maintain a high molecular weight glycol chitin.

**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 micro-constant for the Glu-35 ionization in native lysozyme when Asp-52 is protonated, we can calculate all four micro-constants from the two titration constants \( G_1 \) and \( G_2 \) as was done in Chapters IV and V. This assumption is much 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 \( pK_2 \) will have little consequence. The calculations of the micro-constants have been carried out and are listed in Table 1. Also listed are the titration constants found for Asp-101 and Asp-101'. Because we have shown in Chapter IV that Asp-101 does not interact electrostatically with the active site carboxyls, these titration constants can be taken as the true apparent dissociation constants.

We will discuss the ionization of Glu-35 first. Micro-constant \( pK_3 \), which dominates the ionization of Glu-35, shows the expected 0.5 unit increase when subsite C in Figure 1 is filled by NAG-\( \beta \)CH\(_3\), NAG\(_2\)-\( \beta \)CH\(_3\) or NAG\(_3\). Micro-constant \( pK_2 \) does not show this increase, probably as a result of perturbed binding. Because the
correctness of \( p_{k_2} \) is in doubt and \( p_{k_4} \) is subject to an equally large error (equal and opposite) we will discard these constants from further analysis.

**Glutamic 35.** -- Somewhat surprising is that Glu-35 shows no further change in \( p_{k_3} \) when subsite D as well as C is filled. This result confirms a kinetic observation on the hydrolysis of nitrophenyl-\( \beta(1-4)-2\)-acetamido-2-deoxyglucopyranosyl-\( \beta\)-glucopyranoside, NAG-Glu-\( \beta\)-\( \phi \)NO\(_3\), 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 \( p_{k_3} \) 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* (Parsons et al., 1969). This should dispel the last doubts (Neuberger and Wilson, 1967) that Glu-35 is the general acid functioning in the hydrolytic mechanism.

**Aspartic 52.** -- Aspartic acid residue 52 appears to undergo no shift in its \( p_{k_1} \) upon complexation. The apparent change in \( p_{k_4} \) is probably due to error in \( p_{k_2} \). At first thought, the lack of change in \( p_{k_1} \) when subsites C and D are filled is surprising. As was discussed in Chapter V, this is more understandable if Asp-52 is solvated by its surrounding protein structure. Unfortunately, the
hydrolytically observed pK values for small molecular weight substrates have generally not been determined accurately enough for a meaningful comparison with the properties of Asp-52 determined here and in Chapters IV and V. 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 pk₁ 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 is 4.5 (Parsons et al., 1969).

**Aspartic 101.** -- The pK behavior of aspartic acid residue 101 in native lysozyme follows the expected pattern. NAG-βCH₃ does not interact with the residue. Thus the pK of Asp-101 is 4.3, about the value in the free enzyme. NAG₂-βCH₃ 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 1 indicates that this results in a pK of 3.9. NAG₃ forms the same hydrogen bond plus one more from the ring A acetamido N-H to Asp-101. A further lowering of pK occurs to 3.7, a value in good agreement with the previously determined number. NAG₃-Glu-βCH₃ should interact with Asp-101 in the same manner if it binds as expected to subsite 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. 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 to the derivative (at low pH) as it binds to native lysozyme in a totally different position. 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 NAG₃ 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 studied here and in Chapter IV. The highest concentration of salt which was studied is only slightly below the 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, an observation which confirms other data indicating the essential identity of the crystal and solution structures (Raftery et al., 1968b).

It has been proposed that NAG₃ induces a specific reorientation of Glu-35 when it binds (Blake et al., 1967). This is supported by the lack of change in the Glu-35' pK₂ when NAG₃ binds to the derivative in what must be a slightly distorted position. Were the increase in the Glu-35 pK₃ from 6.0 to 6.5 merely due to nonspecific hydrophobic effects, a similar increase should have been observed in pK₂.
We have shown that the titration behavior of the three carboxyls in lysozyme known to interact with inhibitors and substrates correlates well with other catalytic, binding, and magnetic effects. This does not mean necessarily that all the functionally important titratable groups are now identified and characterized. There are unexplained effects which have been observed (Rand-Meir et al., 1969a; Davies et al., 1969). Although great progress has been made toward understanding lysozyme, the catalog of structural and physical information is not yet complete.
References


Dahlquist, F. W., and Raftery, M. A. (1968), Biochemistry 9, 3277.


Table 1. The Micro-Constants for Asp-52 and Glu-35 and the Dissociation Constants for Asp-101 and Asp-101' in Some Complexes

<table>
<thead>
<tr>
<th>Ionization step</th>
<th>Inhibitor or substrate complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAG-β(CH₃)</td>
</tr>
<tr>
<td>pk₁ (Asp 52)</td>
<td>4.43</td>
</tr>
<tr>
<td>pk₂ (Glu 35')</td>
<td>5.22</td>
</tr>
<tr>
<td>pk₃ (Glu 35)</td>
<td>6.01</td>
</tr>
<tr>
<td>pk₄ (Asp 52)</td>
<td>5.22</td>
</tr>
<tr>
<td>Asp 101</td>
<td>[4.2]⁣</td>
</tr>
<tr>
<td>Asp 101'</td>
<td>[4.2]⁣</td>
</tr>
</tbody>
</table>

\(^a\) Determined at 25°C in 0.15 M KCl.

\(^b\) The microsteps, pk, are identified in Chapter IV, Figure 7. 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.
Figure 1. The difference pH titration in the presence of 122 mg methyl-2-acetamido-2-deoxy-β-D-glucopyranoside at 25°C 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 Chapter IV. The pK value for Asp 101' is for the ester derivative.
with Methyl-\(\beta\)-NAG

Difference, Moles H\(^+\)/Mole Asp 52 Ester

\begin{align*}
pG_1 & = 4.35 \\
pG_2 & = 6.50 \\
\text{Asp 101, } pK & = 4.30 \\
\text{Asp 101', } pK & = 4.10 \\
\text{Glu 35', } pk_2 & = 5.10
\end{align*}
Figure 2. The difference pH titration in the presence of 20 mg methyl-β-chitobioside, at 25°C 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 Chapter IV. The pK value for Asp 101′ is for the ester derivative.
with Methyl-\(\beta\)-Chitobioside

\[
\begin{align*}
\text{pG}_1 & \quad 4.40 \\
\text{pG}_2 & \quad 6.60 \\
\text{Asp} \ 101 \ pK & \quad 3.90 \\
-\text{Asp} \ 101' \ pK & \quad 4.10 \\
-\text{Glu} \ 35' \ pK_2 & \quad 5.20
\end{align*}
\]
Figure 3. The difference pH titration in the presence of 2.5 mg chitotriose at 25°C in 0.15 M KCl. The data require five titration constants for a close fit. The numbers pG₁, pG₂, and pk₂ have the same meaning as in Chapter IV. The pK value for Asp 101' is for the ester derivative.
with Chitotriose

Difference, Moles H⁺/Mole Asp 52 Ester

pGI 4.45
pG2 6.50
Asp 10, pK 3.70
-Asp 10, pK 4.10
-Glu 35, pK 5.10

pH
Figure 4. The difference pH titration in the presence of 20 mg methyl-\(\beta(1-4)\)-chitotriosyl-\(\beta\)-D-glucopyranoside at 25°C 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 Chapter IV. The pK value for Asp 101' is for the ester derivative.
with Methyl β-(1-4)-Chitotriosyl-β-D-Glucopyranoside

- pG₁ 4.40
- pG₂ 6.60
- Asp 10I, pK 3.65
- Asp 10I', pK 4.10
- Glu 35, pk₂ 5.20
Figure 5. The difference pH titration in the presence of 2.5 mg chitotriose at 25°C 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 Chapter IV. The $pK$ value for Asp 101' is for the ester derivative.
with Chitotriose in 0.5M KCl

Difference, Moles $H^+$/Mole Asp 52 Ester

$pG_1$ 4.60
$pG_2$ 6.50
Asp101 $pK$ 3.90
Asp101' $pK$ 4.30
Glu 35 $pK_2$ 5.20

pH
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°C 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 glycol chitin at 25°C in 0.15 M KCl. The apparent pK of Glu 35 is about 8.0 to 8.5.
with Glycol Chitin

Glu 35
\( \sim pK 8.0 - 8.5 \)
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.
<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
<td></td>
<td>Asp 101</td>
<td>Glu 35</td>
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