Lysozyme-catalyzed Reaction of the N-Acetylglucosamine Hexasaccharide

DEPENDENCE OF RATE ON pH*

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SUMMARY

The rate of lysozyme-catalyzed reaction of the β(1 → 4)-linked hexasaccharide of N-acetylglucosamine was determined at 40°, 0.1 ionic strength, and pH 2 to 10.5. Reaction rate was determined by measuring increase in reducing sugar for the hydrolysis reaction or by charcoal column analysis for the transglycosylation reaction. At all pH values, only one bond in the hexasaccharide is cleaved. Apparent first order kinetics is followed at substrate concentrations above \( K_m \) in accord with equally strong binding of hexasaccharide substrate and tetrasaccharide product. Measurements of the hydrolysis reaction above \( 10^{-4} \)M substrate were complicated by transglycosylation to the substrate as acceptor. The pH profile gave for \( k_{cat} \) the kinetic apparent \( pK \) values 3.8 and 6.7 and a maximum value of \( k_{cat} \) 0.15 s\(^{-1}\), and for \( k_{cat}/K_m \) the \( pK \) values 4.2 and 6.1. Equilibrium measurements of hexasaccharide binding at 40° and 0.1 ionic strength showed that \( K_m \) equals \( K_a \) with maximum value \( 9 \times 10^{-3} \)M at pH 5. Detailed interpretation of the pH profiles was carried out under the assumptions that nonproductive complexes are of kinetic importance and the structure of the productive complex and the catalytic mechanism are those suggested by the crystallography. This analysis gave for the free enzyme and nonproductive enzyme-hexamer complex, respectively, the values \( pK \) 6.1 and \( pK \) 6.7 for Glu-35 and \( pK \) 3.4 to 3.7 and \( pK \) 3.8 for Asp-52.

The β(1 → 4)-linked hexasaccharide of N-acetylglucosamine is the most effective of the known substrates of lysozyme. These compounds show values for \( k_{cat} \) (0.1 to 0.5 s\(^{-1}\)) that are four orders of magnitude greater than \( k_{cat} \) for the N-acetylglucosamine trimer (1) or the nitrophenyl glycoside of GlcNAc-Glc (4). X-ray diffraction results (5) indicate that a hexasaccharide should fill the active site cleft, and accordingly a single bond in the hexasaccharide substrates is most rapidly cleaved to give dimer and tetramer products. Thus kinetic tests of mechanistic proposals are appropriately carried out using hexasaccharide substrates.

The pH dependence of lysozyme catalysis has been studied using cell walls (6), oligomers of N-acetylglucosamine (1, 7), glycol chitin (8), and the nitrophenyl glycoside of GlcNAc-Glc (4). In no case was the pH dependence of the kinetic constants shown to follow a simple mechanism. This has led to uncertainty with regard to how well the proposal based on the x-ray data (3) accords with kinetic observation, in particular with regard to participation of the groups Asp-52 and Glu-35.

This report describes kinetic data that define \( k_{cat} \) and \( k_{cat}/K_m \) as a function of pH for the lysozyme-catalyzed hydrolysis of the N-acetylglucosamine hexamer. The data follow over the pH range 2 to 10 a relatively simple mechanism that is consistent with the participation of two ionizable groups in bond rearrangement. The complexities found using other substrates are not exhibited by the hexamer.

MATERIALS AND METHODS

The β(1 → 4)-linked hexamer of N-acetyl-D-glucosamine was prepared from chitin by a modification of the previously published procedure (9), the charcoal-Celite separations were improved by thermostating at 60°; after the initial separation of the hexasaccharide fraction, shallower gradients (15 to 30%, ethanol) were used for rechromatography. All saccharides referred to in this study as hexamer, tetramer, etc., are oligomers of N-acetylglucosamine. Lysozyme was obtained from Worthington Biochemicals. \(^{14}C\)-Labeled monomer was from ICN and had specific activity 1 mCi per mmole. Other chemicals were reagent grade. Deionized water was used.

\( pH \)–pH was measured at room temperature (25 ± 1°) using a Leeds and Northrup or Radiometer PHM26 instrument. Standard buffers were 0.05 M potassium acid phthalate, pH 4.01, and Radiometer S1001, pH 6.50.

Measurement of Reaction Rate—The lysozyme-catalyzed hydrolysis of hexasaccharide was followed using either of two techniques: (a) the increase in reducing groups or (b) column separation of reaction products.

1. Reducing sugar was determined by ferricyanide reaction. Saccharide stock solutions (10\(^{-3}\)M hexasaccharide in buffer) were stored frozen and kept for no longer than 2 weeks. Enzyme
Fig. 1. Record of the change in 420 nm absorbance for an analysis of the lysozyme-catalyzed reaction of (GlcNAc)₆. The symbols A, B, C, and D are for buffer blank, enzyme blank, hexamer blank, and zero-time reaction sample (hexamer and enzyme in buffer, respectively). E, F, G, and H are reaction samples taken after 8, 16, 24, and 35 min. Reaction conditions: (E)₀ = 0.004 mg per ml, (S)₀ = 3 x 10⁻⁶ M, and pH 5.2.

Fig. 2. Charcoal-Celite column chromatographic separation of the products of lysozyme-catalyzed hydrolysis of 1.6 x 10⁻⁴ M (GlcNAc)₆ in the presence of 0.15 M ¹⁴C-labeled monomer at pH 5.0. A through F are the elution positions for monomer through hexamer, respectively. The dashed curve indicates the radioactivity (1%) in the effluent fractions. The solid curve gives the reducing sugar content of the effluent, measured continuously as change in ferricyanide absorbance. The absorbance changes within 2% of the expected value. This correction was taken into account in calculation of the extent of reaction.

pH was controlled in the range 3 to 9 with 0.01 M sodium acetate, phosphate, or carbonate. No buffer was used below pH 3. Ionic strength was brought to 0.1 with NaCl.

2. Reactions carried out for separation on charcoal columns contained 0.15 M N-acetyl-d-glucosamine. This is a sufficiently high concentration of acceptor to drive the lysozyme-catalyzed reaction into transglycosylation (1). Five milligrams of hexamer and 62.5 mg of monomer were dissolved in 2.5 ml of lysozyme in buffer and reacted for 1 hour at 40°. The reaction was stopped by adding 0.1 ml of concentrated HCl. The mixture was separated on a 1:3 Celite-charcoal column (0.6 x 45 cm) thermostatted at 60°. Elution was with a linear gradient of 0 to 90% ethanol over 1 liter at 2 ml per min flow rate. After elution of the HCl, the column was connected to the sample line of the automated analysis system described above, except the reaction bath temperature was 70°. A typical elution pattern is shown in Fig. 2. In the transglycosylation reaction of hexamer with

stock solutions (1 mg per ml of lysozyme in buffer) were made fresh before each experiment and the concentration was determined using E₄₂₀ = 25.5 (10). In a typical experiment, saccharide stock solution was diluted with buffer to give 100 ml of the desired saccharide concentration. Half of this solution was reserved for use as the saccharide blank, and the remaining 50 ml were equilibrated at 40°. Enzyme was added and immediately 25 ml (zero-time sample) were quenched by mixing with sufficient HCl to bring the pH below 2.0. Aliquots of 5 ml were similarly quenched at four times chosen to give extent of hydrolysis covering the range 10 to 70%. Reducing sugar analyses were performed with a Technicon autoanalyzer coupled with a Gilford 2000 recording spectrophotometer. Sample pumped at 1.2 ml per min was mixed with two solutions: 0.005 M K₃(Fe(CN)₆) at 0.42 ml per min; 0.5 M Na₂CO₃, 0.1 M KCN, 0.15 mg Dupanol per ml at 0.16 ml per min. Reagents were prepared fresh and filtered before each experiment. Oxidation was at 90° for 15 min. Because the rate of the ferricyanide-sugar reaction depends on pH, it was necessary to adjust pH of the sample to between pH 10 and 10.5 with a previously determined amount of NaOH solution added immediately before analysis. The change in ferricyanide concentration was determined as the decrease in 420 nm absorbance. Fig. 1 shows the Gilford recorder tracing for analyses of samples from a reaction at 3 x 10⁻⁴ M hexamer.

The change in ferricyanide absorbance is linear in saccharide concentration to at least 2 x 10⁻⁴ M in the sample solution, with a least squares slope (color value) for hexamer, 0.093 ± 0.004 absorbance unit per mole per liter. The reducing power of hexamer is slightly less than that of dimer or tetramer, which are the products obtained through lysozyme-catalyzed hydrolysis. Comparison of hexamer with an equimolar mixture of dimer and tetramer showed that the color value of the products was 2.12 times that of hexamer, i.e. complete hydrolysis of hexamer should result in a decrease in ferricyanide absorbance 2.12 times greater than the decrease for the hexamer reactant. Reactions analyzed before and after exhaustive enzymic hydrolysis gave absorbance changes within 2% of the expected value. This correction was taken into account in calculation of the extent of reaction.
moiomer, the products are pentamer and dimer. 14C-labeled monomer was used in the reaction of Fig. 2 (10^7 cpm total counts in reaction mixture). As expected, label was dominantly incorporated into pentamer. Less than 5% of the label went into other products unless the extent of reaction was greater than 75%, in which case there was significant hydrolysis of the pentamer product. Product concentrations were calculated from areas under the peaks of the effluent pattern using color values determined with standard samples.

pH was controlled in the range 2 to 7 using the same buffers as for hydrolysis reactions. At pH above 7 control was by pH stat. One experiment at pH 2 was with 0.01 M acetate. The data of Fig. 6 are sufficient to exclude specific buffer effects at the 0.01 M level.

Equilibrium Binding—Equilibrium binding of hexamer to lysozyme was measured at 40° using fluorescence and absorbance methods described previously (11). The concentration of protein was 0.02 mg per ml and measurements were begun within 10 s after mixing in order to minimize the effect of hydrolysis. At pH near 5 hydrolysis was sufficiently fast to require extrapolation of fluorescence changes to zero time.

RESULTS

The time course of the lysozyme-catalyzed hydrolysis of hexamer (Fig. 3) is characteristic of a reaction first order in substrate over the ranges of pH and substrate concentration studied in this work. The apparent first order character at substrate concentrations near or above that required for half-saturation of the enzyme is expected (12) from the essentially identical equilibrium binding (13) of the hexamer and one product (tetramer). The second product (dimer) binds almost two orders of magnitude more weakly and does not compete. The rate Equation 1 for product inhibition

\[ v = \frac{k_{\text{cat}}(E) [S]}{K_m(1 + \frac{[S]}{K_p}) + [S]} \]  

reduces to the following first order relationship if \( K_m = K_p \).

\[ v = k_1 [S] \]

Values of \( k_1 \) were calculated using the integrated form of Equation 2. The rate \( v_0 \) for an initial substrate concentration \([S]_0\) was determined from the average of four values of \( k_1 \) measured at times of reaction covering 10 to 70% completion. Values of \( k_1 \) usually deviated by less than 5% from the average, except for pH below 3 or above 7 where rates of reaction were low. The initial rate of reaction could not be determined as accurately as \( k_1 \) because of the high reducing sugar blank associated with substrate. Enzyme concentration was varied for experimental convenience. The rate of reaction was linear in enzyme concentration (0.002 to 0.008 mg per ml).

Values of the kinetic constants \( k_{\text{cat}} \) and \( K_m \) were obtained from computerized least squares fits of the Lineweaver-Burk equation to the data, using appropriate weights (14). Fig. 4 shows data for pH 3, 5.2, and 7. Table I lists values of \( K_m \), \( k_{\text{cat}} \), and related functions. These estimates of the kinetic constants were from data obtained at substrate concentrations in the range 8 \( \times 10^{-5} \) M to 6 \( \times 10^{-4} \) M, within which range there was no deviation from linearity in Lineweaver-Burk plots. The noise level in the reducing sugar assay was 0.3 \( \times 10^{-5} \) M and precluded precise measurements below 8 \( \times 10^{-4} \) M substrate. Apparent substrate inhibition was observed at concentrations above 10^{-4} M (Fig. 4, pH 5.2 data). This inhibition effect is not true inhibition but represents glycosyl transfer in which substrate is both donor and acceptor, i.e. in which the nonreducing terminal 2 units of the hexaasaccharide bind at sites E and F of the glycosyl enzyme. The development of glycosyl transfer at concentrations 10^{-4} to 10^{-3} M accords with estimates (reviewed in Reference 13) of the strength of the interaction between saccharide and this region of the active site.

It is important that in the lysozyme-catalyzed reaction of hexamer the products are tetramer and dimer at low and high pH, as has been shown previously for pH 5 (1). Column analyses of the transglycosylation products formed with 14C-labeled monomer showed no significant radioactivity except in the pentamer component (95 to 98% of the transglycosylation radioactivity was in pentamer at pH 2, 5, and 7).

At pH above 8, high enzyme concentration is required to ob-
obtained in this way from column analyses agree well with those from standard kinetic analyses (Fig. 5).

Thus the measured rate constant $v''$ corresponds to $k_{cat}$. Values of $k_{cat}$ to saturate the enzyme (the added monomer acceptor does not compete with hexamer at the concentrations used). Because monomer is 2000 times more effective than water in the transfer reaction (reviewed in Reference 13), a $pK$ near 8 has been associated with an active site group in a glycol-chitin lysozyme complex (16). It is possible that the larger substrates interact with groups that are outside of the active site defined by the crystallography and that are not important for hexasaccharide hydrolysis. In contrast, the pH dependence of cell wall hydrolysis shows an apparent $pK$ 9 to 10, which is abolished or moved to $pH$ 6 to 7 through modification of the $e$-amino groups (reviewed in Reference 13).

Because the enzyme blank interfered with reducing sugar analysis, the transglycosylation reaction with column analysis of the products was used to determine rates at alkaline $pH$. Rate constants $k_1$ and $a_0$ were calculated as described above, except data were for one single time of reaction. Uncertainties listed are standard deviations. Buffers: A, $HCl$; B, 0.01 M sodium acetate; C, 0.01 M sodium phosphate; D, 0.01 M sodium carbonate. $NaCl$ was added to give ionic strength 0.1.

Reactions were at 0.001 to 0.008 mg of enzyme per ml and were followed to approximately 70% completion, which required about 2 hours at $pH$ between 3 and 7 or 2 to 6 hours at other $pH$ values. Uncertainties listed are standard deviations.

The parameters were determined by least squares fit of the Lineweaver-Burk equation to reaction rates measured at 6 to 10 substrate concentrations ranging from $8 \times 10^{-8}$ to $6 \times 10^{-5}$ M. Reactions were at 0.001 to 0.008 mg of enzyme per ml and were followed to approximately 70% completion, which required about 2 hours at $pH$ between 3 and 7 or 2 to 6 hours at other $pH$ values.

In Fig. 8, $K_m$ is compared with equilibrium binding data for hexamer and trimer. The kinetic constant $K_m$ does not differ significantly from the equilibrium constant $K_s$ measured for hexamer. The appreciable scatter (+20%) in $K_m$ reflects the strong binding of substrate and the inability to measure rates at substrate concentrations significantly below $K_m$. The stronger binding of hexamer compared with trimer (Fig. 8) confirms previous results (17-19). The change in log $K_s$ for hexamer is about

![Fig. 5. Dependence of $k_{cat}$ on $pH$ for the lysozyme-catalyzed hydrolysis of (GlcNAc)$_4$. Symbols carry the same meanings as in Fig. 5.](http://www.jbc.org/)

![Fig. 6. Dependence of log $k_{cat}$ on $pH$ for the lysozyme-catalyzed hydrolysis of (GlcNAc)$_4$. Symbols carry the same meanings as in Fig. 5.](http://www.jbc.org/)

## Table I

| $pH$ | Buffer | $k_{cat}$ (sec$^{-1}$) | $K_m/k_{cat}$ (sec M) |
|------|--------|----------------------|-----------------------|
| 2.0  | A      | 0.0016 ± 0.0005      | 0.1 ± 2.0 x 10$^{-3}$ |
| 2.0  | A      | 0.0027 ± 0.0010      | 11.0 ± 1.0 x 10$^{-3}$ |
| 2.5  | A      | 0.0082 ± 0.0022      | 1.85 ± 0.9 x 10$^{-4}$ |
| 3.0  | B      | 0.0185 ± 0.0010      | 9.1 ± 0.9 x 10$^{-4}$  |
| 3.0  | B      | 0.0214 ± 0.0027      | 9.1 ± 1.8 x 10$^{-4}$  |
| 3.5  | B      | 0.045 ± 0.002        | 2.20 ± 0.31 x 10$^{-4}$|
| 3.8  | B      | 0.080 ± 0.006        | 1.37 ± 0.75 x 10$^{-4}$|
| 4.2  | B      | 0.116 ± 0.005        | 1.30 ± 0.11 x 10$^{-4}$|
| 4.5  | B      | 0.124 ± 0.009        | 7.9 ± 1.5 x 10$^{-5}$  |
| 4.6  | B      | 0.130 ± 0.006        | 6.9 ± 1.2 x 10$^{-5}$  |
| 4.8  | B      | 0.140 ± 0.005        | 4.7 ± 0.6 x 10$^{-5}$  |
| 4.9  | B      | 0.138 ± 0.004        | 8.3 ± 0.5 x 10$^{-5}$  |
| 4.9  | B      | 0.134 ± 0.005        | 4.8 ± 0.7 x 10$^{-5}$  |
| 5.2  | B      | 0.141 ± 0.006        | 8.4 ± 0.8 x 10$^{-5}$  |
| 5.2  | B      | 0.145 ± 0.003        | 7.4 ± 0.4 x 10$^{-5}$  |
| 5.2  | B      | 0.144 ± 0.005        | 5.9 ± 0.6 x 10$^{-5}$  |
| 5.2  | B      | 0.141 ± 0.006        | 5.6 ± 0.8 x 10$^{-5}$  |
| 5.6  | B      | 0.143 ± 0.007        | 6.3 ± 0.8 x 10$^{-5}$  |
| 5.6  | C      | 0.158 ± 0.005        | 6.1 ± 0.7 x 10$^{-5}$  |
| 6.0  | C      | 0.122 ± 0.007        | 1.25 ± 0.15 x 10$^{-4}$|
| 6.5  | C      | 0.086 ± 0.011        | 1.83 ± 0.47 x 10$^{-4}$|
| 7.0  | C      | 0.039 ± 0.002        | 4.7 ± 0.4 x 10$^{-4}$  |
| 7.5  | C      | 0.0189 ± 0.0012      | 9.7 ± 1.1 x 10$^{-4}$  |
| 8.0  | C      | 0.0071 ± 0.0007      | 3.8 ± 0.5 x 10$^{-3}$  |
| 9.0  | D      | 0.00068 ± 0.00008    | 1.85 ± 0.45 x 10$^{-2}$|
0.1 in pK and 2,000 in kcat/Km.

The substrate is understood to bind to the enzyme to give a productive complex and one or more nonproductive complexes. The productive complex is the reactant in the rate-determining step. Because the hexasaccharide is cleaved at only one glycosidic bond to yield tetramer and dimer, this substrate forms a single productive complex. Nonproductive complexes are all other enzyme-substrate forms that are present at steady state concentrations comparable to or larger than the concentration of the productive complex. There may be more than one non-productive complex and these may be on the path to the productive complex or off this path but in equilibrium with free enzyme and substrate. Nonproductive binding of substrate is well established. The original proposal was based (a) upon the crystallographic data (5) and (b) upon the large increases in rate that are associated with increased substrate chain length but not paralleled by stronger equilibrium binding of substrate (1).

Recent dye displacement (17) and fast reaction studies (18) have established that in the equilibrium binding of hexasaccharide several complexes are formed at comparable concentrations. One of these may be the productive complex.

The mechanism of Equation 3 implies that in the steady state, productive and nonproductive complexes are in rapidly established equilibrium with enzyme and substrate. The equivalence of Km and Ks (Fig. 8) supports this assumption. Fast reaction studies (18) have shown that these equilibria involve more than one step.

“Appendix” Equations 4 to 8 give expressions derived from the mechanism of Equation 3 for the pH dependence of log kcat, log Km, and log Kcat/Km. Use of these functions in developing a physically reasonable explanation of the pH dependence of the lysozyme-catalyzed reaction requires consideration of other information, in particular the x-ray structures given by Phillips and his colleague (5). These results indicate that four ionizable groups (the side chains of glutamic acid 35 and aspartic acids 52, 66, and 101) are affected by saccharide binding. In the enzymic mechanism proposed from the crystal structure Asp-52 is understood to function as an electrostatic catalyst with its negative charge stabilizing the developing carbonium ion, and Glu-35 functions as a general acid catalyst in proton transfer to the glycosidic oxygen. Asp-101 participates in saccharide binding. Asp-66 is not in contact with bound saccharide but is in part of the protein that changes structure in the binding reaction. Previous work (reviewed in Reference 13) has shown that in the free enzyme Asp-101 has pK 4.2 to 4.7; Asp-66, pK less than 2; Asp-52, pK 3.0 to 4.6; and Glu-35, pK 6.0 to 6.5. Binding of saccharides that resemble substrate lowers the pK of Asp-101 by 0.6 to 0.8, raises that of Glu-35 by 0.4 to 0.6, and has no significant effect on Asp-52. Because the pK of Asp-66 is below 2, this group need not be considered in explanation of the present kinetic data.

The data of Figs. 6 and 7 closely follow expectation for the dependence of the rate parameters on ionizations of apparent pK 3.8 and 6.7 for kcat and pK 4.2 and 6.1 for Kcat/Km. A usual interpretation of such pH profiles is that the first pair of apparent pK describe ionizations of groups in the enzyme-substrate complex that are important for catalysis, and the last pair describe ionizations of the same groups in the free enzyme. This simple interpretation cannot hold for lysozyme. The crystallographic results suggest that Asp-101 interacts similarly with trimer and hexamer, and perturbation of Asp-101 needs to be taken into account in explaining the pH dependence of kcat and Kcat/Km.

Since other ionizations in the pH range 2 to 11 should not be affected by substrate binding, only Asp-101 and the two catalytic groups Glu-35 and Asp-52 need be considered.
Because the pK of Asp-101 is nearly 2 units below the pK of Glu-35, the alkaline limb of the pH-rate profile should be simply interpretable as reflecting Glu-35. In agreement, the kinetic apparent pK values of 6.1 and 6.7 are expected from previous equilibrium measurements (13) on ionization of Glu-35 in the free protein and in saccharide-enzyme complexes, respectively.

The acid limb of the pH rate profile contains the contribution of Asp-101. The kinetic apparent pK 4.2 estimated from $k_{on}/K_s$ data is within experimental error equal to that found for Asp-101 in the free enzyme. Examination of Equation 8 of the “Appendix” shows that because of this equality the equilibrium pK of Asp-52 in the free enzyme is equal to the pK of Asp-101 in the productive complex. If the pK of the latter group is the same in the productive hexamer-enzyme complex as in the trimer-enzyme complex, the equilibrium pK of Asp-52 is approximately 3.4 in the free enzyme. However, between pH 2 and 5 the change in enzyme-trisaccharide association, which has been attributed to perturbation of Asp-101, is slightly greater than that for hexasaccharide. The 0.3 unit smaller change in log $K_s$ for hexasaccharide might reflect either a smaller perturbation of Asp-101 in the hexasaccharide-enzyme complex compared with the trimer-enzyme complex, or a partially compensating perturbation of Asp-52. In the former case the pK of Asp-52 in the free enzyme would be approximately 3.7.

The following arguments indicate that the contribution of Asp-101 to the pH dependence of $k_{on}$ is likely to be small. (a) The last term of Equation 7 is zero if the perturbation of Asp-101 is, as suggested by the crystal structure, not significantly different in productive and nonproductive complexes. (b) The penultimate term of Equation 7 also should be negligible. The same term appears in Equation 6 which describes the pH dependence of log $K_s$. The pH dependence of the binding of the tetramer of N-acetylglucosamine to lysozyme is essentially identical with that for hexamer (19, 20). However, the tetrasccharide is cleaved only slowly by lysozyme, and there can be no appreciable concentration of productive complex. Thus either the pH dependence of hexasaccharide binding is similarly dominated by the behavior of nonproductive complexes or ionizations are similar in productive and nonproductive complexes. On the basis of these arguments one concludes that the kinetic apparent pK for Asp-52 estimated from the $k_{on}$ data is likely to be not significantly different from the equilibrium pK for this group, i.e. Asp-52 has pK 3.8 in the nonproductive enzyme-substrate complex.

It is now possible to compare the values estimated for the pK of Asp-52 using the above analysis of the kinetic data with values estimated from equilibrium measurements. The discussion of the preceding two paragraphs gives, for Asp-52, pK 3.4 to 3.7 in the free enzyme and pK 3.8 in the nonproductive enzyme-substrate complex. Four different kinds of equilibrium experiments have been understood to bear upon the pK of Asp-52. (a) Asp-52 is the closest ionizable group to the region of the active site where monosaccharides bind. The free energy of binding is invariant with respect to pH over the range 2 to 5 for N-acetylglucosamine (21) and its β-methylglycoside (22), and the pH dependence of the binding of oligosaccharides over the range 2 to 5 has been interpreted in terms of perturbation only of Asp-101. Thus the near equality of the pK of Asp-52 in free enzyme and nonproductive complex found in the kinetic analysis accords with the conclusions from equilibrium measurements. (b) Calorimetric experiments (23) have shown that the enthalpy of binding of β-methyl-N-acetylglucosaminide, unlike the free energy, varies strongly with pH. The enthalpy decreases by 1.6 kcal between pH 2 and 6, and the change follows an ionization of apparent pK 3.8. It is difficult to explain this change except in terms of Asp-52, and the associated pK agrees well with the estimate from the kinetic data. (c) Parsons and Raftery (16) have estimated pK values for Asp-52 in the free enzyme and in several enzyme-saccharide complexes, through experiments in which they compare the hydrogen ion-binding properties of the native protein with those of a lysozyme specifically esterified at Asp-52. They concluded that the apparent pK of Asp-52 in the free protein is 4.5 and that Asp-52 is not perturbed through saccharide binding. Sharon (24) has obtained similar results with an ester of lysozyme formed with an epoxide analog of the substrate. These pK estimates differ significantly from those of the kinetic analysis. (d) A pK near 4.7 was found in the pH dependence of binding of the α anomer of N-acetylglucosamine (25, 26), and this was assigned to Asp-52. In these measurements the effect of pH is small (a change of 0.2 unit in log $K_{obs}$) and the binding process is complicated by the presence of two distinguishable enzyme-α anomer complexes.

Because the estimate of the pK of Asp-52 from the kinetic analysis differs considerably from the estimates made from two equilibrium approaches, it is appropriate to consider the assumptions behind the kinetic analysis. These are: (a) nonproductive complexes are kinetically important; (b) all enzyme forms before the rate-limiting step are in rapidly established equilibrium; (c) Glu-35 and Asp-52 participate in catalysis; (d) the environment and perturbation of Asp-101 in the enzyme-trimer complex, which is the complex for which there are crystallographic data, is the same as in the productive enzyme-hexamer complex; (e) no other ionizable groups of pK 2 to 11 are important for catalysis or binding. Assumptions a and b are supported by experiment and have been discussed. Assumptions c through e are central in the proposal from the crystallography. Assumptions d and e are equivalent to asserting the correctness of the proposed structure for the enzyme-hexasaccharide complex that was developed by extension of the structure of the enzyme-trisaccharide complex. A pK near 4.5 for Asp-52 in the free enzyme can be reconciled with the kinetic data by relaxing assumptions d or e. For example, if the model for the productive complex based upon the crystallography is wrong, Asp-101 might be perturbed differently in the productive complex than in the enzyme-trimer complex. A pK near 4.5 for Asp-101 in the productive complex, compared with pK 3.4 in the trimer complex, would account for the kinetic data and retain pK 4.5 for Asp-52. This and other explanations consistent with pK 4.5 for Asp-52 represent considerable violations of the structural models. It seems as likely that the equilibrium data that have been analyzed to give an estimate of pK 4.5 for Asp-52 require more complex interpretation. It is possible that reaction of Asp-52 to form the ester leads to structural changes that are reflected in perturbation of other ionizations. In this regard trimer binds less strongly to the ester than to native lysozyme (16). Furthermore, correctness of the assumptions used in analysis of the kinetic data is supported by the agreement between the kinetic and equilibrium estimates for the apparent pK of Glu-35 and by the agreement of one equilibrium estimate of the pK of Asp-52 (23) with the estimate from the kinetic analysis.

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From the mechanism of Equation 3 we can write for the observed rate constant:

\[ k_{\text{cat}} = \frac{k_p}{1 + K_{\text{np}}^p} \cdot k_p \]  
(4)

The rate constant (kp) for breakdown of the productive complex is assumed, following the crystallographic suggestion, to depend on the fractional ionization (\( \alpha \)) of two groups, with Group 2 (Glu-35) required in the protonated form for catalysis and Group 1 (Asp-52) in the deprotonated form.

\[ k_p = k_{\text{max}} \cdot a_{\text{ES},p}^1 \cdot (1 - a_{\text{ES},p}^2) \]  
(5)

The pH dependence of \( \log K_s \) is given in Equation 6.

\[ -\log K_s = \log (K_{\text{p,ref}} + K_{\text{np,ref}}) + \log \left(1 + R_{1,1} \frac{(1 - a_{\text{ES},p}^1)}{(1 - a_{\text{ES},p}^2)}\right) \]  
(6)

The association constants \( K_{\text{p,ref}} \) and \( K_{\text{np,ref}} \) are for equilibrium binding under the reference condition of very low pH. The products are over all \( n \) ionizable groups affected by binding of substrate. The pH dependence of \( \log k_{\text{cat}} \) and \( \log k_{\text{cat}}/K_m \) are obtained by combining Equations 4 to 6 and are given in Equations 7 and 8.

\[ \log k_{\text{cat}} = \log(k_{\text{max}} \cdot R \cdot K_{\text{p,ref}}^1 \cdot K_{\text{np,ref}}^1) + \log a_{\text{ES},p}^1 + \log \left(1 + R_{1,1} \frac{(1 - a_{\text{ES},p}^1)}{(1 - a_{\text{ES},p}^2)}\right) \]  
(7)

\[ \log k_{\text{cat}}/K_m = \log k_{\text{cat}}/K_s = \log(k_{\text{max}} \cdot K_{\text{p,ref}}^1 \cdot K_{\text{np,ref}}^1) + \log (1 - a_{\text{ES},p}^1) + \log \left(1 + R_{1,1} \frac{(1 - a_{\text{ES},p}^1)}{(1 - a_{\text{ES},p}^2)}\right) \]  
(8)

The constants \( K^1 \) are proton dissociation constants for Group 1. The last terms of Equations 7 and 8 introduce the contribution of groups important for substrate binding but not for catalysis.

As for the simple Michaelis-Menten mechanism, the free-enzyme pK of Groups 1 and 2 are reflected in the pH dependence of \( k_{\text{cat}}/K_m \). With regard to the pH profile for \( k_{\text{cat}} \) it is of interest that in the situation where nonproductive complexes are kinetically important (R small), (a) the pH dependence is dominated by ionizations in the nonproductive complex, and (b) the pH dependence can be due entirely to groups that are not important for catalysis but are perturbed differently in productive and nonproductive complexes. The second point bears importantly upon the present data. The pH dependence observed for \( k_{\text{cat}} \) does not require that two groups (Glu-35 and Asp-52) participate in catalysis, although the data are consistent with such participation. It is possible that one or both groups are not catalytically important but are strongly perturbed (to pK outside the 2 to 11 range) in forming the productive complex.

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