Productive and Unproductive Lysozyme–Chitosaccharide Complexes. Kinetic Investigations†

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ABSTRACT: Flow and relaxation methods were used to study the kinetics of oligosaccharides binding to lysozyme and the pre-steady-state kinetics of the lysozyme-catalyzed, hydrolysis of chitohexose. The minimal mechanism demonstrated, and the kinetic parameters pertaining to the elementary steps, allow interpretations of previous equilibrium and steady-state kinetic measurements which had yielded only complex constants, reflecting both productive and unproductive lysozyme–substrate complexes. In contrast to previous assumptions, the data presented in this paper provide evidence for "stable" productive lysozyme–substrate complexes. Our proposed mechanism utilizes structural information and accounts for the difference in efficiency of lysozyme-catalyzed hydrolysis of chitopentose and chitohexose.

Knowledge of the crystal structure of hen egg-white lysozyme and of various lysozyme–inhibitor complexes (Phillips, 1966, 1967; Blake et al., 1967a,b), together with solution studies (Rupley and Gates, 1967; Rupley et al., 1967; Chipman et al., 1968; Pollock and Sharon, 1970; Parsons et al., 1969; Dahlquist and Raftery, 1969; Imoto et al., 1972), have led to a proposed mechanism of action for this enzyme. The active site of lysozyme can accommodate six units of a polymeric substrate, which make contact with sites of interaction on the enzyme, designated sites A–F. Only those saccharide molecules whose pyranose rings make contact with sites D and E are catalytically hydrolyzed (Figure 1). Catalytic rate enhancement is, in part, the result of distortion associated with the binding of a substrate saccharide ring to site D. The free energy requirement for distortion results in a proportion of the substrate binding exclusively to sites A–C of the enzyme, i.e., in an unproductive binding mode (Figure 1).

Interpretation of steady-state and equilibrium measurements (Imoto et al., 1972) which reflect productive and unproductive complexes, depends on a knowledge of the concentration of various complexes.

In the preceding paper (Holler et al., 1975) we described the use of a chromophoric lysozyme inhibitor, Biebrich Scarlet, to determine the concentration of both productive and unproductive lysozyme–substrate complexes. Here we will describe kinetic investigations of the elementary steps associated with the formation of both types of complexes using temperature-jump and stopped-flow methods. Kinetic investigations of the lysozyme-catalyzed hydrolysis of the $\beta(1 \rightarrow 4)$-linked hexasaccharide of *N*-acetylglucosamine are also reported. Among the lysozyme substrates of known structure, the hexamer is hydrolyzed with greatest efficiency giving, as the only products, chitotetrose and chitobiose (Rupley and Gates, 1967). A preliminary report of some of these investigations has appeared (Rossi et al., 1969; Holler et al., 1969, 1970).

Experimental Procedures

Materials

Enzyme. Twice-crystallized lysozyme (lots LY 8 HB and LY 9 AA, activities 8800 and 10,000 units/mg) and three times crystallized lysozyme (lots LYSF 9 BD and LYSF 9 FM, activities 12,000 and 10,600 units/mg) were obtained from Worthington Biochemical Corporation. No difference between these preparations was observed. Concentrations were determined spectrophotometrically from the absorbance at 280 nm using a value for ϵ_M of 38,400 M^{-1} cm^{-1}.

Substrate and Inhibitors. The $\beta(1 \rightarrow 4)$-linked oligosaccharides of *N*-acetylglucosamine, chitobiose, chitotriose, chitotetrose, chitopentose, and chitohexose were isolated from hydrolyzed chitin following the method of Rupley (1964), and further purified (Holler et al., 1975).

Indicators. p-Nitrophenol (lot 722708) (Fisher Scientific Co.) was twice crystallized from toluene before use (mp 113.6°, uncorr.). Chlorophenol Red (Allied Chemical Corp.) was used without further purification. Salts and buffers were reagent grade. Water was distilled, deionized, boiled, and cooled to room temperature under nitrogen before use.

Apparatus

The temperature-jump apparatus (Eigen and De Maeyer, 1963) used in the relaxation experiments has been described (Kresheck et al., 1966). The change in absorbance at 420 and 550 nm was observed using *p*-nitrophenol and Chlorophenol Red, respectively, as pH indicators. The light source was a tungsten–iodine lamp. The wavelength was selected by placing a filter between the light source and the temperature-jump cell. The 420-nm filter (24% transmittance at 420 nm, half-band width 6.5 nm) was obtained from Baird-Atomic Inc., and the 550-nm filter (30% transmittance at 550 nm, half-band width 9.0 nm) from Spectrum System Inc. Changes in absorbancy were recorded on a Tektronix storage oscilloscope. Those changes exhibited by the indicator due to a temperature-dependent change in its ionization state which occurred in less than 10 μsec were

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grounded out. A jump in temperature of 9° was achieved by discharge of an appropriate capacitor. The reaction cell was thermostated within ±0.2° of the initial temperature.

A Durrum–Gibson stopped-flow spectrophotometer was used. Changes in the absorbancy of a pH indicator were observed using a 2-cm reaction cell. The cell had a dead-time of 15 msec, as determined by Havsteen's method (1969). For fluorescence measurements the apparatus was modified. The regular tungsten–iodine lamp was replaced by a high intensity mercury–xenon lamp. A 0.7-cm cell with 5-mm windows was used to enhance the intensity of the fluorescent light. The cell had a dead-time of 15 msec. Excitation light of 294 nm was obtained by use of the regular Durrum monochromator. Light scatter was reduced by a cutoff filter (Durrum, No. 2; 1% transmittance at 300 nm, 75% transmittance at 320 nm) on the emission side.

Cary Model 14 Recording Spectrophotometer. Slow reactions, such as the hydrolysis of chitohexose, were also measured on a Cary Model 14 recording spectrophotometer, equipped with a 0–0.1 absorbance unit slidewire, using pH indicators. The cell compartments were flushed with nitrogen during the measurements to minimize absorption of carbon dioxide by the solutions.

An Hitachi-Perkin-Elmer MPF2 spectrofluorimeter with 3 mm × 10 mm quartz cells was used to make fluorescence measurements. The longer light path was in the excitation beam of 290 nm. The shorter light path was used for measurements of emitted light at 320 nm.

A Radiometer or Corning pH meter was used to determine the pH of solutions.

Methods

Reactions were followed by observing either the change in absorbance of a pH indicator or of the fluorescence of the lysozyme tryptophans. The changes in hydrogen ion concentration of the reaction, and in the tryptophan fluorescence have been investigated previously (Rupley and Gates, 1967; Parsons and Raftery, 1970; Lehrer and Fasman, 1967; Teichberg and Sharon, 1970).

When indicators were used, stock solutions of lysozyme (0.02–0.3 m*M*) and chitosaccharides (0.1–100 m*M*) were dissolved in solutions either 0.1 *M* in Na_2SO_4 or 0.3 *M* in NaCl. Na_2SO_4 was used only in temperature-jump relaxation experiments. Stopped-flow measurements indicated no difference in the data obtained in the presence of one or the other salt. Initial concentrations of indicators were 0.1–0.4 m*M* *p*-nitrophenol and 0.5 m*M* Chlorophenol Red. Fluorescence was measured in the presence or absence of sodium–potassium phosphate buffer (pH 5–8) or sodium acetate buffer (pH 2–5) with sufficient NaCl to give a final ionic strength of 0.3 *M*. In a few experiments an ionic strength of 0.1 *M* was also used and the kinetic parameters were found to be the same, within experimental error. All stock solutions were filtered through 0.45-μ Millipore filters.

Temperature-Jump Relaxation Experiments. The reaction mixture was prepared before the experiments except when chitohexose or chitopentose was used. The solutions were adjusted to the desired pH with 1 *N* NaOH or HCl and placed in the temperature-jump cells. After the solutions had come to the desired temperature, the relaxation experiments were started. The measurements were repeated several times. The experimental results were recorded only when no change in pH was observed at the end of the experiment. This technique was used with chitobiose, chitotriose, and chitotetrose.

In experiments with chitohexose, which is hydrolyzed more rapidly than the other sugars, a different procedure was followed. The pH of the stock solutions was adjusted to the desired value; 100 μl of the chitohexose stock solution at the desired temperature was mixed in the temperature-jump cell with 2.5 ml of enzyme solution, at the desired temperature and pH. Measurements were initiated less than 10–20 sec after mixing the hexasaccharide with enzyme. The pH of the solution after mixing was checked with a glass microelectrode.

Initial concentrations were 0.015–0.35 m*M* lysozyme, and 0.015–0.45 m*M* chitotriose, chitotetrose, or chitohexose. Chitotriose was studied at pH 6.4–7.5, chitotetrose at pH 7.0, and chitohexose at pH 7.5.

Stopped-Flow Experiments. Nearly all experiments were performed with saccharide in excess of enzyme. Control experiments were run for all measurements. In these, similar solutions were used but with either enzyme or substrate absent. In experiments in which one of the reactants was in excess over the other, the experiments were repeated using the reverse condition. Data were recorded only when the same results were obtained under both conditions. The kinetics of the reactions were also measured in the reverse direction. In measurements of the α process, solutions of enzyme and sugar, at saccharide concentrations equal to the dissociation constant of the saccharide–lysozyme complex, were placed in one syringe and mixed in the stopped-flow apparatus with a tenfold excess of an appropriate buffer solution from the other syringe.

A different procedure was used to reverse the β process since the latter is not observed with chitotriose but only with higher oligosaccharides. Enzyme–saccharide solutions (tetramer, pentamer, or hexamer) were mixed in the stopped-flow apparatus with a large excess of chitotriose. The initial concentrations in the displacement reactions were 0.015–0.1 m*M* lysozyme, 0.07–0.15 m*M* displaceable saccharide, and 1–7 m*M* chitotriose. The reversal gave the predicted changes in absorbance of the indicator or in lysozyme fluorescence, and the observed rate constants were in agreement with those predicted from investigations of the reaction in the forward direction.

In the usual stopped-flow experiments, the concentration of lysozyme in one syringe was 0.0006–0.2 m*M* and of saccharide in the other syringe 0.002–5 m*M*. At the beginning of the measurements, the solutions were mixed in a 1:1 ratio.

Experiments using pH indicators were performed between pH 5.5 and pH 7.5. Indicator was present in both syringes of the stopped-flow apparatus. The pH of the solutions was adjusted with 1 *N* NaOH before mixing and checked again after mixing. Fluorescence measurements were made between pH 2 and pH 8.

Changes in absorbance observed with the stopped-flow apparatus had to be calculated from the observed changes in light intensity using the following equation: ΔA , log $[(I - \Delta I)/I]$, or when $\Delta I \ll I$, $\Delta A = -0.43(\Delta I/I)$. ΔI denotes the change in intensity during the reaction and I the final intensity.

The Hydrolysis of Chitohexose. Hydrolysis of chitohexose could be observed when the product, chitobiose, was released and bound to excess enzyme. The binding of the dimer to the enzyme is associated with an uptake of protons (Rupley, 1967; Imoto et al., 1972) and with a measurable change in fluorescence intensity (Lehrer and Fasman, 1966, 1967). The initial concentration of lysozyme was 0.5–0.8

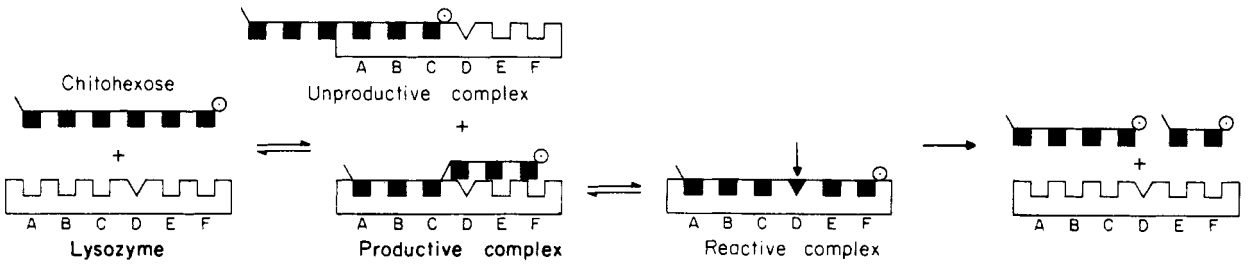

FIGURE 1: Graphical representation of the formation of the two main lysozyme-chitosaccharide complexes considered in the analysis of the data. The unproductive complex involves binding of the reducing end of the substrate (▀◉) to site C of the enzyme and the adjacent pyranose rings bind to sites B and A and then protrude into the solvent. The productive complex involves binding of the nonreducing end of the substrate (◉▀) to site A of lysozyme and the adjacent pyranose rings bind to sites B and C and then protrude into the D-F region of the molecule. The chair conformation of the saccharide is indicated by (⊓), the half-chair conformation by (▼). The reactive complex involves stabilization of the half-chair conformation of the pyranose ring which makes contact with site D by the geometry of this binding site.

m*M*, which is above the value of the dissociation constant for chitobiose binding. In these experiments the concentration ratios of enzyme and substrate could not be reversed since the measurements depend on chitobiose produced in the reaction binding to free enzyme. However, in these experiments, enzyme concentrations were varied to ascertain that the catalytic reaction was not perturbed by the formation of enzymic dimers (Sophianopoulos, 1969). The transmittance changes observed in the experiments were less than 4% and were directly proportional to hydrogen ion concentration.

Evaluation of Kinetic Parameters. Over 90% of each reaction followed apparent first-order kinetics and apparent first-order rate constants were evaluated from appropriate plots of the oscilloscope traces. For each set of reactants and concentrations, at least four measurements were evaluated. The values for the reciprocal relaxation time, or for the observed rate constant, were determined as a function of the concentration of the reactant (Holler et al., 1969, 1970). When the reactant concentrations were comparable, the equilibrium concentrations of the reactants were calculated for a set of assumed overall dissociation constants of the particular process, and the data were plotted using the linear forms of eq 2 and 3. The parameters of the equations were evaluated by a computer program which used iteration to give the best fit of the data to a straight line, as judged by standard deviations of the experimental data (Figure 3a, inset).

The time-dependent fluorescence changes also followed first-order kinetics. A log plot of $A_\infty - A_t$ as a function of $(t + \Delta t)$, where Δt is the dead-time of the instrument (15 msec), gives as an intercept the total signal change of the observed process.

Cary Model 14 Recording Spectrophotometer Measurements of Hydrogen Ion Uptake by Lysozyme. The hydrogen ion uptake by lysozyme which accompanies the binding of chitooligosaccharides at pH 6.3 and 25° was also measured using the Cary spectrophotometer and pH indicators. At zero times, 25 μl of the saccharide solution was added to the sample cell which contained 2 ml of solution 0.1-0.5 m*M* in lysozyme, 0.3 *M* in NaCl, and either 0.15 m*M* in *p*-nitrophenol or 0.05 m*M* in Chlorophenol Red. The absorbance changes are in reference to identical solutions from which lysozyme was omitted. In the experiments with chitohexose, absorbance changes were extrapolated to the time the solutions were mixed. Absorbancy changes for pH indicators were converted into changes in hydrogen ion concentration by means of a calibration curve. The latter was obtained by adding 10-μl aliquots of 5×10^{-4} *N* HCl to solutions which were identical with those used in the experiments except that the sugars were omitted.

Hydrolysis of chitohexose was also measured using the Cary spectrophotometer and pH indicators. Initial concentrations were 0.17-0.85 m*M* lysozyme for the hydrolysis of the chitohexose and 0.05-0.2 m*M* saccharide. Either 0.15 m*M* *p*-nitrophenol or 0.05 m*M* Chlorophenol Red was used as an indicator. The adjustment of the pH of the solutions, and of the ionic strength, was as described for the stopped-flow experiments. The changes in absorbance were measured with reference to solutions which contained all components of the solutions in the sample cell except the saccharide. The reactions were initiated by injecting 10-100 μl of saccharide into 2.0 ml of enzyme and immediately mixing the solutions with the syringe needle. Quartz cells with 1.0-cm path length were used.

In order to compare the spectrophotometric measurements with those obtained in stopped-flow experiments, the data had to be corrected since the two instruments have different light path lengths.

Results

Kinetics of Lysozyme-Saccharide Interactions. The binding modes of chitohexose which are considered in our analysis of the kinetic data are shown in Figure 1. Unproductive complexes are produced when saccharide binds at the A-C sites of the enzyme, with the reducing end at site C. In this binding mode the units at the nonreducing end of chitotetrose and higher oligomers protrude into the solvent (Holler et al., 1975). Also shown in Figure 1 are stable, productive complexes in which the saccharides also fill the A-C sites. However, in this binding mode, the nonreducing end of the saccharide occupies site A and the reducing end units protrude into the D-F region of the enzyme (Holler et al., 1975).

The absorbance changes shown in Figure 2 are produced when chitohexose binds to lysozyme and perturbs the hydrogen ion equilibria of the enzyme. At least two different processes are seen. One process occurs in the 5-msec time region (Figure 2a), and will be referred to as the α process. It is observed with chitotriose and larger oligomers under conditions where chitotriose is known to bind exclusively to the A-C sites of the enzyme (Imoto et al., 1972; Holler et al., 1975). The α process is considered to be associated with the formation of unproductive lysozyme-substrate complexes. The other process, which will be referred to as the β process, occurs in the 100-msec time region (Figure 2b). It is observed only with oligomers larger than chitotriose and is considered to be associated with formation of productive complexes.

When the initial substrate concentration $[S_0]$ is much

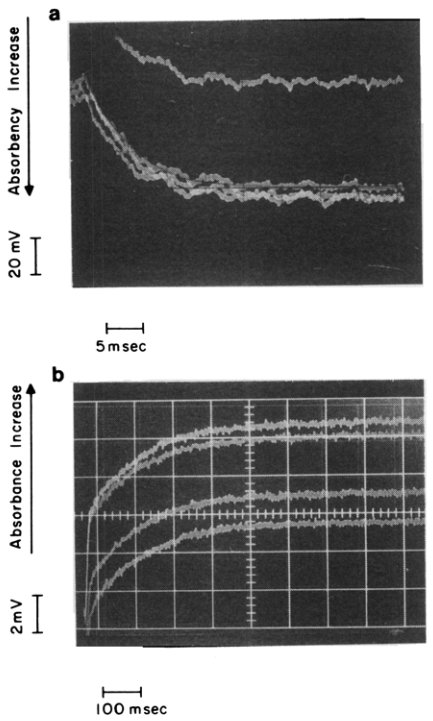

FIGURE 2: The α and β processes observed in the binding of chitohexose to lysozyme. Photographs of oscilloscope traces recording the change in absorbance of *p*-nitrophenol at 420 nm during a stopped-flow kinetic experiment; pH 6.3, 0.1 *M* sodium sulfate. For each process, four consecutive reactions were recorded to demonstrate the reproducibility. In order to display four experiments on the oscilloscope screen, part of the initial fast change in transmission was grounded out in some of the experiments. (a) α process at 20°. The transmittance of the reaction mixture is 1.2 V. Initial concentrations were 0.09 m*M* lysozyme, 0.22 m*M* chitohexose, and 0.15 m*M* *p*-nitrophenol. (b) β process at 25°. The initial fast jump of the oscilloscope trace is due to the α process. Initial concentrations were 0.14 m*M* lysozyme, 0.35 m*M* chitohexose, and 0.25 m*M* *p*-nitrophenol.

higher than the initial enzyme concentration $[E_0]$, the reactions follow a single exponential rate law. The concentration dependence of the observed rate constant, k_{obsd}, for the α process is consistent with a two-step reaction scheme in which the first step reaches equilibrium and the second step is comparatively slow. The same is true for the β process:

$$EU_2^{\alpha} \underset{k_{32}^{\alpha}}{\overset{k_{23}^{\alpha}}{\rightleftharpoons}} EU_1^{\alpha} \overset{K_{12}^{\alpha}}{\rightleftharpoons} E + S \overset{K_{12}^{\beta}}{\rightleftharpoons} ES_1^{\beta} \underset{k_{32}^{\beta}}{\overset{k_{23}^{\beta}}{\rightleftharpoons}} ES_2^{\beta} \quad (1)$$

In this equation EU_1^{α} and EU_2^{α} represent the unproductive lysozyme–substrate complexes, and ES_1^{β} and ES_2^{β} the productive complexes.

The dependence on reactant concentration of the observed rate constant, k_{obsd} (determined in stopped-flow experiments), and of the relaxation time, τ (determined in temperature-jump experiments), for the α process is as follows (eq 2, Brandt et al., 1967; eq 3, Amdur and Hammes, 1966; Hammes, 1968):

$$k_{obsd} = k_{32}^{\alpha} + \frac{k_{23}\phi_{\alpha}[S_0]}{[S_0] + K_{12}^{\alpha}\phi_{\alpha}} \quad (2)$$

$$\tau_{\alpha}^{-1} = k_{32}^{\alpha} + \frac{k_{23}^{\alpha}\phi_{\alpha}[\bar{E} + \bar{S}]}{[\bar{E} + \bar{S}] + K_{12}^{\alpha}\phi_{\alpha}} \quad (3)$$

In these equations $\bar{E}$ and $\bar{S}$ refer to the equilibrium concentrations of the reactants. By analogy the appropriate equations for the β process can be written, in which k_{23}^{β} and K_{12}^{β} are multiplied by ϕ_{β}. The terms ϕ_{α} and ϕ_{β} arise because both productive and nonproductive complexes are considered to involve the A–C sites of the enzyme (Holler et al., 1975) and, therefore, cannot be formed simultaneously:

$$\phi_{\alpha} = K_{12}^{\beta}/(K_{12}^{\alpha} + K_{12}^{\beta}) \quad (4)$$

$$\phi_{\beta} = K_{13}^{\alpha}/(K_{13}^{\alpha} + K_{12}^{\beta}) \quad (5)$$

The dissociation constants for the complexes are defined in the following way:

$$K_{12}^{\alpha} = [E][S]/[EU_1^{\alpha}] \quad (6)$$

$$K_{13}^{\alpha} = [E][S]/[EU_2^{\alpha}] = K_{12}^{\alpha}K_{23}^{\alpha} \quad (7)$$

$$K_{12}^{\beta} = [E][S]/[ES_1^{\beta}] \quad (8)$$

The concentration dependence of the reciprocal relaxation time for the α process observed in the formation of lysozyme–chitotriose, –chitotetrose, and –chitohexose complexes was measured in temperature-jump experiments at 39°, pH 7.5 (Figure 3a). A pH indicator was used to monitor the reaction. The concentration dependence of the observed rate constant for the α process seen in the formation of lysozyme–chitotriose and –chitohexose complexes was measured in stopped-flow experiments at 10°, pH 7.5 (Figure 3b). In these experiments the reaction was monitored by measuring changes of the enzyme fluorescence. The data were evaluated using linear forms of the rate equations (2) and (3) (Figure 3a, inset). The parameters were found to be independent of the method used to observe and measure the reaction (Table I). The values for K_{13}^{α} determined in kinetic studies and in equilibrium determinations (Holler et al., 1975) are also in good agreement (Table I).

The concentration dependence of the observed rate constant for the β process observed in the formation of lysozyme–chitohexose complexes was measured in stopped-flow experiments at pH 6.3, 20° (Figure 4a). The data points obtained in the same experiments for the α process are shown for comparison. A pH indicator was used to measure the reaction. The data were evaluated using a linear form of eq 2 in which k_{23}^{β} and K_{12}^{β} are multiplied by ϕ_{β} (Figure 4a, inset). The values of the parameters are listed in Table I. The β process was also monitored in stopped-flow experiments in the pH region 5.0–7.0 by measuring changes in the enzyme fluorescence (Figure 4b). In these experiments the rate constant, k_{32}^{β}, for the formation of ES_1^{β} from ES_2^{β} was obtained directly (Figure 4b). Under comparable conditions good agreement between the rate constants was obtained regardless of the method of measurement (Table I). The equilibrium measurements of the overall dissociation constant for productive binding of chitohexose to lysozyme (Holler et al., 1975) and the overall dissociation constant for the β process K_{13}^{β}, calculated from the kinetic parameters, are also in reasonable agreement (Table I).

The data, which are consistent with the minimum mechanism shown in eq 1, in which the isomerization of enzyme complexes is proposed, may also pertain to a mechanism which involves isomerization of the free enzyme (eq 9).

$$E_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E_2 + S_0 \overset{K_s}{\rightleftharpoons} ES_1 \quad (9)$$

When $S_0 \gg E_0$, the concentration dependence of the observed rate constant for this process is

$$k_{obsd} = k_{12} + [k_{21}K_s/(K_s + S_0)] \quad (10)$$

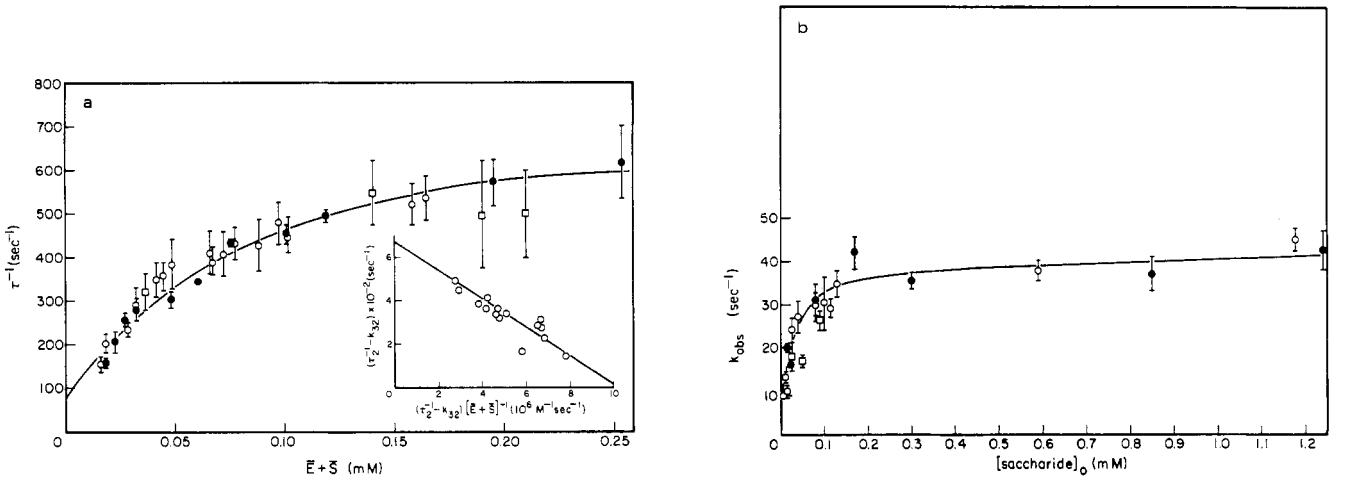

FIGURE 3: (a) The concentration dependence of the reciprocal relaxation time at pH 7.5 for the α process observed in the formation of lysozyme–saccharide complexes, measured at 39° in temperature-jump experiments. The reactions were followed by intensity changes at 420 nm in the presence of 0.14 mM p-nitrophenol and 0.1 M sodium sulfate. The solid line was computed from the kinetic constants listed in Table I. Each point represents the average of at least four determinations. The experimental errors are given as mean deviations. Initial concentrations were 0.05–2.0 mM for lysozyme and 0.02–3.5 mM for chitooligosaccharides. (□) Chitotriose; (○) chitotetrose; (●) chitohexose. In the experiments with chitohexose, the solutions containing lysozyme and saccharide were equilibrated at the initial temperature separately and adjusted to pH 7.5. The solutions were mixed 10 sec before the temperature jump. From a knowledge of the rate constant for the hydrolysis of the saccharide and a value for K_{13}^{α} of 1×10^{-5} M obtained in equilibrium measurements (Holler *et al.*, 1975), it is calculated that under the most unfavorable conditions, less than 10% of the substrate was hydrolyzed before the measurements of the relaxation time. Inset: Some of the data for the α process of chitotetrose evaluated according to a linearized form of the analytical rate equation (eq 3). The line was established by a computer program which calculated k_{23}^{α} (intercept) and k_{12}^{α} (slope) by iteration of k_{23}^{α} to give the best fit of the data to a straight line. The values of the equilibrium concentrations were calculated using the K_{13}^{α} values listed in Table I. (b) The concentration dependence of the observed rate constants for the α process observed in the formation of lysozyme–saccharide complexes with chitotriose (□ and ●) and chitohexose (○) measured at pH 7.5 and 10° in stopped-flow experiments. The reactions were followed by changes in fluorescence (○ and ●) and intensity changes at 420 nm in the presence of 0.1 mM p-nitrophenol (□). In fluorescence measurements the excitation wavelength was 294 nm and a cutoff filter was used on the emission side (Durrum No. 2; 1% transmittance at 300 nm, 75% transmittance at 320 nm). The solid curve was computed from the kinetic constants listed in Table I. Each point represents the average of at least four determinations. The experimental errors are given as mean deviations. The kinetic parameters were evaluated using a linearized form of the analytical rate equation and a computer program.

Table I: Parameters Pertaining to Formation of Productive and Unproductive Lysozyme–Chitosaccharide Complexes.[a]

Observation		pH	Temp (°C)	k_{23} (sec^{-1})	k_{32} (sec^{-1})	K_{12} (μM)	K_{23} (μM)	K_{13} (μM)
α process chitotriose	T, NP	7.5	38	400 (380)[b]	50	70 (110)[b]	0.13	9 (15)[c]
α process chitotriose	T, NP	7.0	27	200	20	70	0.10	7 (16)[d]
α process chitotriose	T, NP	6.7	38	680	70	150	0.10	15
α process chitotriose	T, NP	6.4	38	750	60 (28)[f]	180	0.08	14 (6–10)[e]
α process chitotetrose	T, NP	7.0	38	750	70	91	0.09	8
α process chitohexose	T, NP	7.5	38	600	70	70	0.10	7
α process chitohexose	S, NP	6.3	25	240	20	80	0.08	6
β process chitotetrose	S, NP	6.3	26	21	5	6	0.2	1.2 (9.4)[e]
β process chitotetrose	S, NP	6.3	35	35	15	8	0.5	4
β process chitohexose	S, NP	6.3	25	11	4	15	0.4	6 (6)[e]
β process chitohexose	S, NP	6.3	35	46	15	9	0.33	3 (15)[g]

[a] Rate and equilibrium constants for the α and β processes. Abbreviations used are T for temperature-jump relaxation technique, S for stopped-flow technique, and NP when p-nitrophenol was used as indicator. Standard deviations are generally not better than 10% for k_{23}, 50% for k_{32}, and 50% for the dissociation constants when the temperature-jump relaxation technique was used. Similar standard deviations are obtained in the stopped-flow measurements. A somewhat larger variation of the parameters was observed when experiments were repeated from time to time. For definitions of equilibrium constants see eq 6–8. [b] pH 8, 25° (Pecht et al., 1970). [c] 31° (Rupley et al., 1967). [d] 25° (Lehrer and Fasman, 1966). [e] pH 5.0, 25° (Dahlquist et al., 1966). [f] pH 6.0, 28.7° (Chipman and Schimmel, 1968). [g] pH 5, 40° (Rupley and Gates, 1967).

Equation 10 predicts that k_{obsd} decreases with increasing substrate concentrations until it reaches a plateau value equal to k_{12}. Previous results (Chipman and Schimmel, 1968; Holler et al., 1969) and the data presented here, however, show an increasing k_{obsd} value with increasing substrate concentrations.

Measurements of the time-dependent changes in hydrogen ion concentration and in fluorescence associated with lysozyme–chitohexose interactions indicate a third slow process, the γ process, in the 1–10-sec time region (Figure 5a). This process is observed only with the tetramer, pentamer, and hexamer. The value of the apparent first-order rate constant for this process could not be determined with accuracy because the signal was weak. When the concentrations of sugar was much larger than that of the enzyme, and was varied between 0.05 and 3 mM, k_{obsd} for the γ process appeared to be independent of saccharide concentration. One explanation of this observation is that the forward rate

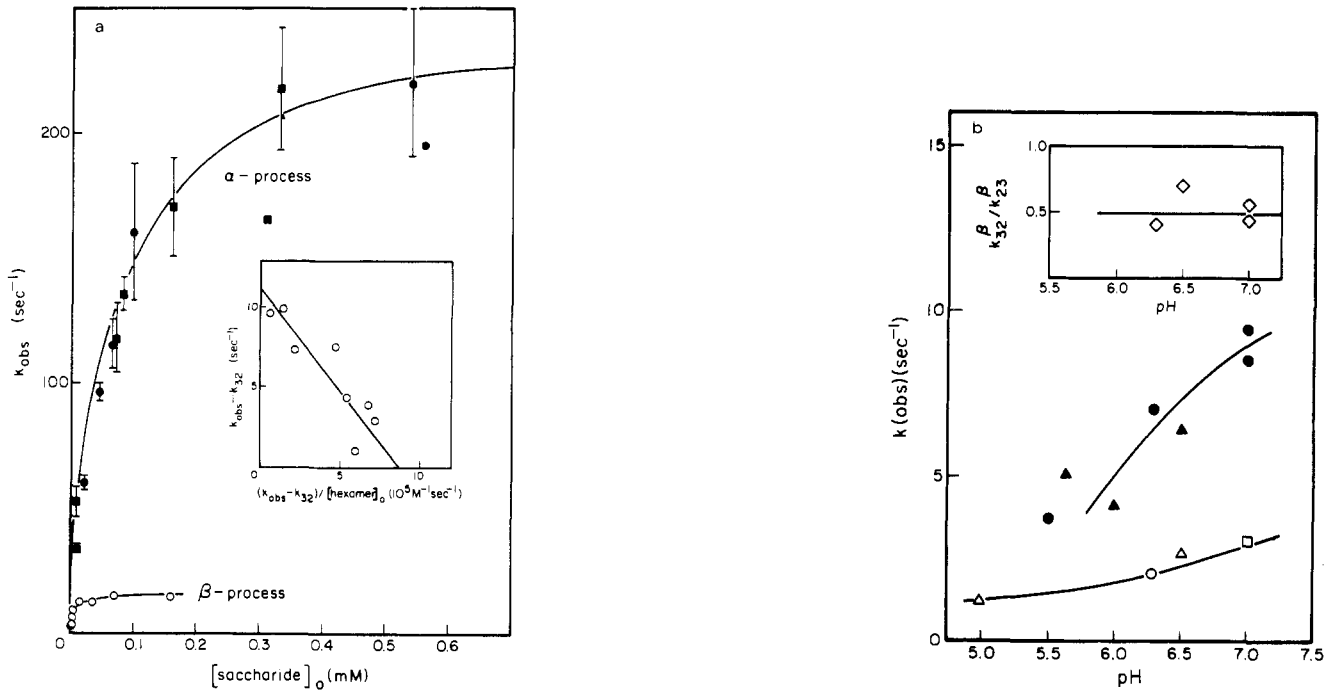

FIGURE 4: (a) The concentration dependence of the observed rate constants for the β process observed in the formation of lysozyme–chitohexose complexes measured in stopped-flow experiments at pH 6.3, 20°. The reactions were followed by intensity changes at 420 nm in the presence of 0.14 mM p-nitrophenol and 0.1 M sodium sulfate. The solid lines show the concentration dependence of the k_{obsd} values of the β process as computed from the kinetic constants listed in Table I. Each point represents the average of at least four determinations. Initial concentrations were larger for the saccharides than for lysozyme (0.003–0.03 mM) except in the two single experiments where the enzyme was in excess. For comparative purpose the data points for the α process obtained in the same experiments are also shown. Inset: This is part of the evaluation procedure for the data of the β process according to a linearized form of the analytical rate equation (eq 2) (Holler et al., 1969, 1970). The values of the parameters pertaining to the two processes are listed in Table I (see also the Experimental Procedures). (b) The observed rate constant for the β process as a function of pH at 16.5°. Measurements were performed in the stopped-flow apparatus with observation of the fluorescence changes. The symbols refer to chitotetrose (△), chitopentose (□), and chitohexose (○). The filled symbols refer to conditions where saccharide concentrations were larger than K_{12}^{β}. The value for k_{obsd} under these conditions is approximately equal to the sum of the rate constants for the interconversion of ES_1^{β} to ES_2^{β} (eq 2), $k_{obsd} \approx k_{32}^{\beta} + k_{23}^{\beta}$. The open symbols refer to rate constants of the back reaction, the formation of ES_1^{β} from ES_2^{β}. In these experiments a mixture of the enzyme and chitosaccharide was mixed with a solution which contained chitotriose at a concentration much higher than the concentration of the saccharide in the enzyme mixture. The value for k_{obsd} refers under this condition to k_{32}^{β} (see Experimental Procedure). This procedure allows evaluation of the formation and dissociation rate constants of ES_2^{β}, k_{23}^{β}, and k_{32}^{β}. Inset: This contains values for the ratio of these rate constants which is equal to the equilibrium constant of the β process, $ES_1^{\beta}/ES_2^{\beta}$. Initial concentrations were 0.01 mM lysozyme, and chitotetrose, chitopentose, and chitohexose were 0.3 mM for the measurement of $(k_{32}^{\beta} + k_{23}^{\beta})$. Sodium–potassium phosphate or sodium acetate was used as a buffer. The ionic strength was adjusted to 0.2 M by addition of sodium chloride.

constant for formation of ES^{γ} is smaller than the reverse rate constant. The experimental data consistent with this suggestion are that the values of K_{13}^{β} for the three oligosaccharides (determined in equilibrium and kinetic experiments) are of comparable magnitude (Table I), indicating that the formation of ES^{γ} does not contribute to the stability of the complex. The k_{obsd} value increases approximately threefold in going from reactions involving chitotetrose to those involving the hexamer.

The Cleavage of Chitohexose. Figure 5b illustrates the time-dependent uptake of hydrogen ions by lysozyme associated with cleavage of chitohexose. The reaction can be followed because a signal change occurs when chitobiose liberated in the reaction binds to lysozyme.

The data in Tables II and III identify the hydrogen ion uptake in kinetic experiments as being due to the cleavage of chitohexose and specifically to the liberation of chitobiose. Addition of equal concentrations of chitotetrose or chitohexose to lysozyme produces the same amount of hydrogen ion uptake by the enzyme, 0.2 ± 0.02 mol of hydrogen ion per mol of enzyme at pH 6.3, 25° (Table II). Table II shows that the time-dependent hydrogen-ion uptake during lysozyme-catalyzed hydrolysis of chitohexose is exactly the same as the instantaneous hydrogen ion uptake produced by addition of chitobiose to the chitotetrose–lysozyme complex, as long as the molar concentrations of enzyme and saccharides are the same in both experiments.

The lysozyme-catalyzed cleavage of chitohexose was also measured by changes in the enzyme fluorescence which accompany this hydrolysis. The data in Table IV identify those fluorescence changes which are due to the cleavage of chitohexose and specifically to liberation of chitobiose. Mixing chitotetrose and chitohexose with the enzyme gave essentially similar fluorescence signal changes (Table IV). The time-dependent fluorescence signal change observed in the lysozyme-catalyzed hydrolysis of hexamer is essentially the same as that produced by addition of chitobiose to the chitotetrose–lysozyme complex, providing that the molar concentration of enzyme and saccharides are the same in both experiments (Table IV).

The time-dependent fluorescence changes which accompany the lysozyme-catalyzed hydrolysis of chitohexose at pH 7.0, 25°, are shown in Figure 6. The fluorescence signal changes follow a single exponential rate law for over 90% of the reaction, as is expected for a simple breakdown of the reactive enzyme–chitohexose complex. Under comparable conditions good agreement between rate constants was obtained regardless of whether the hydrolysis was followed by fluorescence changes of the enzyme or hydrogen ion uptake by the enzyme (Table V). In order to compare our data

Table II: The Hydrogen Ion Uptake by Lysozyme Which Accompanies the Binding of Chitooligosaccharides Measured in the Cary 14 Spectrophotometer, pH 6.3, 25°.

Chitooligosaccharide	Concn of Chitooligosaccharide (m*M*)	Indicator[a]	$(\Delta H^+)/E_0$
Chitotetrose[b]	1.5	p-NP	0.18
	0.3	p-NP	0.18
	1.5	p-NP	0.19
	0.3	CPR	0.17
	0.46	p-NP	0.19
	0.23	p-NP	0.20
	0.46	p-NP	0.20
	0.35	p-NP	0.24
	0.35	p-NP	0.23
		Av	0.20 ± 0.01
Chitohexose[b,c]	0.36	CPR	0.19
	0.09	CPR	0.19
	0.34	CPR	0.20
	0.85	p-NP	0.21
	0.28	p-NP	0.18
	0.28	p-NP	0.18
	0.17	p-NP	0.18
	0.17	p-NP	0.18
	0.36	p-NP	0.22
	0.36	p-NP	0.22
	0.21	p-NP	0.22
	0.22	p-NP	0.22
	0.60	p-NP	0.23
	0.07	p-NP	0.23
		Av	0.20 ± 0.03

[a] p-NP, *p*-nitrophenol; CPR, Chlorophenol Red. [b] Lysozyme is 1 to 0.5 × 10^{-4} *M*. [c] The mixing time was less than 10 sec, a time interval involving less than 10% hydrolysis of the substrate. The listed values are corrected for this hydrolysis which was measured in independent experiments (Table V).

Table III: The Hydrogen Ion Uptake by Lysozyme Which Accompanies the Catalytic Hydrolysis of Chitohexose, and the Binding of Chitobiose to the Chitotetrose–Lysozyme Complex at pH 6.3, 22°.[a]

Initial Concn	Component Added	ΔA (absorbancy units)
0.87 m*M* enzyme	0.38 m*M* chitohexose	0.007 ± 0.001[b]
0.87 m*M* enzyme 0.38 m*M* chitotetrose	0.38 m*M* chitobiose	0.007 ± 0.001[c]

[a] The hydrogen ion uptake was measured with the 0–0.1 absorbancy slidewire at 550 nm in the presence of 0.05 m*M* Chlorophenol Red–0.3 *M* NaCl. In the experiment with chitohexose, the time-dependent signal changes were extrapolated to the time of mixing lysozyme with substrate. [b] Average of 12 determinations of the time-dependent hydrogen ion uptake at t_∞. [c] Average of 10 determinations of the instantaneous hydrogen ion uptake.

with earlier measurements of Rupley and Gates (1967), the hydrolysis step was measured at pH 5.0 and 40° (Table V). The observed rate constant, 0.09 ± 0.01 sec^{-1}, agrees well with the value of 0.14 sec^{-1} determined by steady-state measurements (Rupley and Gates, 1967; Imoto et al., 1972).

Discussion

The kinetic data will be considered in terms of the lysozyme–chitohexose complexes shown in Figure 1 and eq 1. (1) The observation of two distinct kinetic processes, the α and β process, is consistent with equilibrium measurements which indicated the existence of productive and unproductive lysozyme–substrate complexes (Holler et al., 1975). (2) The kinetic data (Table I) are consistent with equilibrium measurements, which indicated that unproductive complexes which occupy the A–C sites compete for these sites with the productive complexes. In the chitohexose–lysozyme binding processes the α process appears as a preequilibrium step for the β process. The latter will not affect the measured parameters of the α process if the two processes involve overlapping substrate-binding sites and the value of ϕ_α approaches 1 (eq 2). ϕ_α will approach 1 if $K_{12}^{\beta} > K_{12}^{\alpha}$ (eq 4). For chitohexose binding this condition is consistent with equilibrium measurements which indicate that chitohexose binds by a factor of three better unproductively than productively (Holler et al., 1975). (3) The observation that the β and γ processes are observed with chitohexose but not with chitotriose is consistent with the suggestion that the stable productive complexes involve not only contacts with the A–C sites but intrusion of the saccharides into the D–F sites. This deduction is consistent with experiments with Biebrich Scarlet, a competitive inhibitor of the lysozyme-catalyzed hydrolysis of cell walls of *Micrococcus lysodeikticus*. Biebrich Scarlet is displaced from lysozyme by chitohexose but not by chitotriose (Holler et al., 1975). The equilibrium data suggested that Biebrich Scarlet interacts only with the F site of lysozyme (Holler et al., 1975). (4)

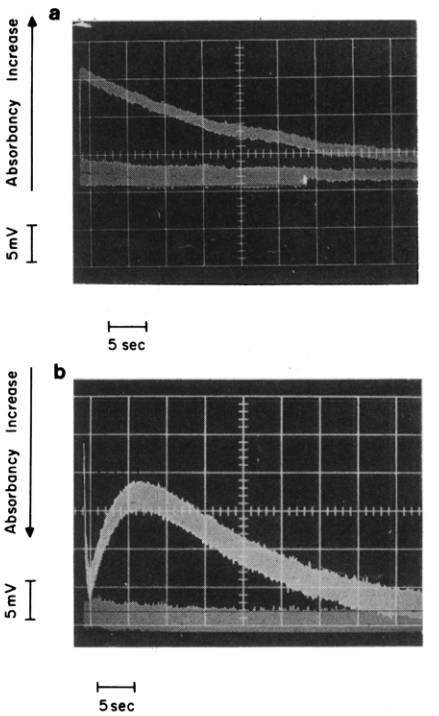

FIGURE 5: The γ process, and the release of chitobiose during the cleavage of chitohexose by lysozyme observed in stopped-flow experiments. The oscilloscope traces follow the reaction-dependent absorbance of *p*-nitrophenol at 420 nm and Chlorophenol Red at 550 nm. (a) The γ process for chitohexose in the presence of excess saccharide at pH 6.3, 25°. The initial jump of the trace corresponds to the α and β processes. Initial concentrations were 0.12 m*M* lysozyme, 0.35 m*M* chitohexose, 0.1 m*M* *p*-nitrophenol, and 0.1 *M* sodium sulfate. (b) The γ process and the release of chitobiose during the cleavage of chitohexose at pH 6.4, 40°. The first sharp absorbance increase refers to the α and β processes. The following decrease in absorbance belongs to the γ process and the final broad increase to the cleavage process. Initial concentrations were 0.05 m*M* Chlorophenol Red, 0.3 *M* sodium chloride, 0.6 m*M* lysozyme, and 0.2 m*M* chitohexose. The release of chitobiose is made visible as a result of binding to free enzyme. The binding is associated with an uptake of protons, which is seen here (see the Results).

Table IV: The Fluorescence Changes Which are Associated with the Binding of Chitobiose, Chitotetrose, and Chitohexose to Lysozyme at pH 6.4, 25°, 0.1 *M* Phosphate Buffer.[a]

Experimental Condition	Final Saccharide Concn	Signal Changes (arbitrary units)		k_{obsd} (sec^{-1})
		Initial Change	Time-Dependent Change	
0.55 m*M* enzyme	0.13 m*M* hexamer	16	8	0.029
Added at end of measurement	0.13 m*M* dimer	6.0		
0.55 m*M* enzyme	0.13 m*M* tetramer	16		
Added at end of measurement	0.13 m*M* dimer	7.5		
0.55 m*M* enzyme	0.13 m*M* tetramer	16.5		
Added at end of measurement	0.13 m*M* dimer	7.5		
1.1 m*M* enzyme	0.13 m*M* hexamer	6.5	5.5	0.027
Added at end of measurement	0.13 m*M* dimer	4.0		
Added an additional amount of dimer	0.26 m*M* dimer	5.0		
1.1 m*M* enzyme	0.13 m*M* hexamer	6.5	7.0	0.020
Added at end of measurement	0.13 m*M* dimer	7.0		
Added an additional amount of dimer	0.26 m*M* dimer	5.5		
1.1 m*M* enzyme	0.13 m*M* tetramer	6.8		
Added at end of measurement	0.13 m*M* dimer	6.5		
1.1 m*M* enzyme	0.13 m*M* tetramer	8.0		
At end of measurement	0.13 m*M* dimer	6.7		
At end of measurement	0.26 m*M* tetramer	8.0		
At end of measurement	0.39 m*M* tetramer	7.0		

[a] Excitation, 290 nm, 6-mm slit; emission, 320 nm, 8-mm slit.

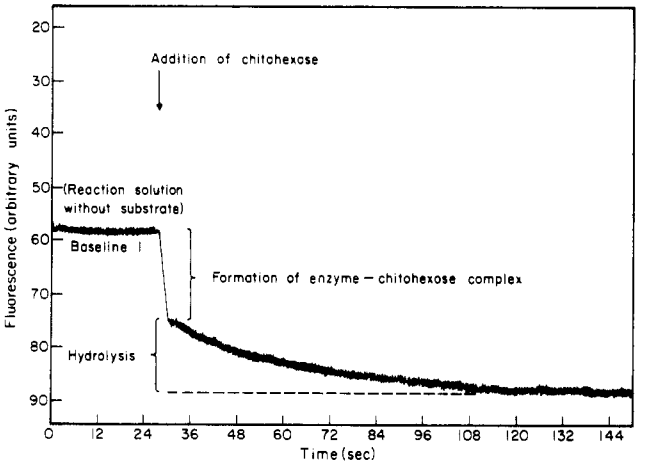

FIGURE 6: The change in fluorescence intensity which accompanies the lysozyme-catalyzed hydrolysis of chitohexose at pH 7.0, 25°, 0.1 *M* phosphate buffer. The initial concentrations were 0.55 m*M* lysozyme and 0.13 m*M* chitohexose (10 μl of hexamer solution, 3 mg/300 μl injected into a final volume of 610 μl). The signal change was measured with a Hitachi-Perkin-Elmer MPF 2 instrument. Excitation at 390 nm, 6-mm slit; emission at 320 nm, 8-mm slit; 10 mm × 3 mm quartz cuvets. The graph represents a trace of the instrument output.

Table V: The Temperature Dependence of the Observed Rate Constant for the Lysozyme-Catalyzed Hydrolysis of Chitohexose, with $E_0 > S_0$.[a]

pH	Temp (°C)	Observation[d]	k_{cat} (sec^{-1})
6.3[b]	20	Chlorophenol Red, S, C	0.011 ± 0.001
	22	Chlorophenol Red, C	0.015 ± 0.002
	24	Chlorophenol Red, C	0.013 ± 0.001
		p-Nitrophenol, C	
	30	Chlorophenol Red, C	0.031 ± 0.002
	31	Chlorophenol Red, S	0.036 ± 0.005
		p-Nitrophenol, S	
		Fluorescence, S	
	35	Chlorophenol Red, C	0.046 ± 0.005
	39	Fluorescence, S	0.066
	40	Chlorophenol Red, C, S	0.067 ± 0.010
5.0[c]	15	Fluorescence, S	0.015 ± 0.003
	20	Fluorescence, S	0.026 ± 0.004
	25	Fluorescence, S	0.047 ± 0.008
	39	Fluorescence, S	0.058
	40	Fluorescence, S	0.090 ± 0.010

[a] Lysozyme, 0.5–0.9 m*M*; chitohexose, 0.1–0.2 m*M*. [b] 0.1 *M* phosphate buffer. [c] 0.2 *M* acetate buffer. [d] Abbreviations: S, stopped-flow; C, Cary Model 14 spectrophotometer.

The parameters associated with the β process are the same for the three oligosaccharides larger than chitotriose (Table I) indicating that the β process does not reflect the chain length of these saccharides. This is consistent with previous evidence (Holler et al., 1975) that the stability of the productive binding mode depends mainly on interactions with sites A–C (Figure 1). The parameters pertaining to the minimal mechanism for the lysozyme-catalyzed hydrolysis of chitohexose at pH 6.3 and 25° are summarized in eq 11.

The existence of a covalent chitotetrose–lysozyme intermediate in the lysozyme-catalyzed hydrolysis of chitohexose is suggested by transglycosidation experiments and other evidence (Imoto et al., 1972). The kinetic data (Figure 6; Table V) indicate that such an intermediate does not accumulate in detectable concentrations in the catalytic reaction. Accumulation of such an intermediate would mean the

$$EU_2^{\alpha} \underset{k_{32}^{\alpha} = 20\ sec^{-1}}{\overset{k_{23}^{\alpha} = 240\ sec^{-1}}{\rightleftharpoons}} EU_1^{\alpha} \underset{}{\overset{}{\rightleftharpoons}} \quad K_{12}^{\alpha} = 8 \times 10^{-5} M$$

$$E + S \underset{K_{12}^{\beta} = 11 \times 10^{-5} M}{\rightleftharpoons} ES_1^{\beta} \underset{k_{32}^{\beta} = 4\ sec^{-1}}{\overset{k_{23}^{\beta} = 11\ sec^{-1}}{\rightleftharpoons}}$$

$$ES_2 \underset{k_{34}^{\gamma} \simeq 0.06\ sec^{-1}}{\overset{k_{43}^{\gamma} < 0.06\ sec^{-1}}{\rightleftharpoons}} ES^{\gamma} \xrightarrow{(k_{cat} = 0.01\ sec^{-1})} E + P \qquad (11)$$

$$ES^{\gamma} \rightleftharpoons ES^* \xrightarrow{k_f}$$

observation of two reaction steps, a fast step due to formation of a chitotetrose–lysozyme intermediate with concomitant liberation of chitobiose followed by the slower hydrolysis of the intermediate. The observation of only a single step in the pre-steady-state kinetic investigations of the lyso-

zyme-catalyzed hydrolysis of chitohexose (Figure 6) indicates either that such an intermediate has formed before the measurements were begun or that it does not accumulate in detectable concentrations in the catalytic reaction. The data in Tables III and IV indicate that the intermediate has not formed before measurements were made since all the signal changes observed in equilibrium measurements of the binding of chitobiose to lysozyme are also seen in the kinetic measurements of chitohexose hydrolysis. The identity of the values of the first-order rate constant observed in our experiments with the steady-state kinetic parameter, k_{cat} (Table V), indicate, therefore, that k_{cat} measures the liberation of both products of the catalytic reaction. The interpretation of pre-steady-state and steady-state kinetic parameters applying to another hydrolytic enzyme and pertinent to this discussion have been published in detail (Himoe et al., 1967; Hess, 1971). The conclusions reached from the analysis of the data presented here are consistent with the results of other workers (Imoto et al., 1972). Dahlquist et al. (1969) concluded that considerable carbonium ion character is involved in the hydrolysis of the synthetic substrate phenyl 4-*O*-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-β-D-glucopyranoside, and that if this intermediate collapses into a covalent enzyme–substrate compound it does so after the rate-determining step.

The minimal mechanism for lysozyme-catalyzed reactions (eq 11) leads to a rational, if speculative, explanation of the experiments of Rupley and Gates (1967) which indicate that the steady-state kinetic parameter K_m for the lysozyme-catalyzed hydrolysis of chitopentose and chitohexose is the same, but that chitohexose is cleaved much more efficiently than chitopentose. If one assumes that ES^{γ} represents the reactive but energetically unfavorable lysozyme–substrate complex (Imoto et al., 1972), the observed velocity of the catalytic reaction is expected to be directly proportional to its concentration. The additional interactions which chitohexose can make with lysozyme, as compared to chitopentose, are expected to lead to a higher concentration of ES^{γ}, and, therefore, to a higher rate of hydrolysis. Since ES^{γ} does not contribute to the measured dissociation constants of the chitopentose- or chitohexose–lysozyme complexes, the observed dissociation constants for the two complexes will be the same.

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