KINETICS OF LYSOZYME-SUBSTRATE INTERACTIONS[1]

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A mechanism for lysozyme-catalyzed hydrolysis of linear polymers of N-acetylglucosamine (GlcNAc), proposed by Phillips and co-workers (Blake, Johnson, Mair, North, Phillips and Sarma, 1967) is based on the determination of the conformation of the crystalline enzyme and of lysozyme-saccharide complexes (Blake, Johnson, Mair, North, Phillips and Sarma, 1967; Blake, Mair, North, Phillips and Sarma, 1967), on model building, and on chemical data (Canfield, 1963; Jollès, Jauregui-Adell, Bernier and Jollès, 1963; Rupley and Gates, 1967; Rupley, Butler, Gerring, Hartdegen and Pecararo, 1967; Vernon, 1967). There is a cleft in the lysozyme molecule which can accommodate six pyranose rings of the substrate at sites designated A, B, C, D, E and F. According to the proposed scheme, the substrates bind preferentially in an unproductive mode to sites A-C of the enzyme. Bond breaking is believed to occur between sites D and E. In order for the substrate to fit into this region of the molecule, the pyranose ring at site

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D must first be distorted from the chair to the half-chair conformation, a process that facilitates cleavage of the $\beta(1\text{-}4)$ glycosidic bond between saccharide units. This distortion is accompanied by an unfavorable free energy change (Rupley and Gates, 1967; Rupley et al., 1967). We have recently succeeded in determining (Rossi, Holler, Kumar, Rupley and Hess, 1969) that the trimer and hexamer of GlcNAc are held to the bond-breaking site with dissociation constants of 10^{-2} M and 10^{-5} M, respectively. We also observed that the hydrogen ion uptake by lysozyme observed at low substrate concentrations is associated with binding to the A-C site, while hydrogen ion release is observed upon subsequent substrate binding to the bond-breaking site. It is therefore possible to obtain additional information about the relationship between structure of substrate and its binding in a productive or unproductive mode from kinetic investigations of the elementary steps involved in this process.

In this paper we report results of kinetic investigations of the binding of the trimer of GlcNAc to the A-C site of lysozyme at pH 7.0, and of the dimer at pH 6.0 and pH 7.0. Measurements were made by the temperature jump method (Eigen and DeMaeyer, 1963), with use of pH indicators to detect the binding process. With this information, we hope to be able later to investigate the kinetics of productive binding of substrates, which involves, in the case of good substrates such as GlcNAc hexamer, the A-C site as well as the bond-breaking site.

We found that at pH 6.0 a single relaxation process is observed (τ= 70 to 1,000 μsec) when solutions containing lysozyme, di-GlcNAc and p-nitrophenol (as indicator) are perturbed. The relaxation time (see Figs. 1 and 2, solid lines) was found to decrease with increasing concentrations of reactants at low reactant concentrations, but to approach concentration independence at higher levels of di-GlcNAc. These observations are characteristic of reactions with the mechanism

$$E + S \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} ES_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ES_2 \qquad (1)$$

(Eigen and De Maeyer, 1963; Amdur and Hammes, 1966). When there is rapid equilibration of the first step

$$\tau_2^{-1} = k_{32} + \frac{k_{23}(\bar{E} + \bar{S})}{\bar{E} + \bar{S} + K_{S_1}} \qquad (2)$$

where $K_{S_1} = k_{21}/k_{12}$. The equation may be written in linear form:

$$[\tau_2^{-1} - k_{32}] = k_{23} - \frac{[\tau_2^{-1} - k_{32}]}{(\bar{E} + \bar{S})} K_{S_1} \qquad (3)$$

The data in Fig. 1 inset, measured at pH 6.0 for the di-GlcNAc system, are plotted according to this relation. The line was established by a computer calculation giving the best linear fit of the data; computed values of k_{23} and K_{S_1} correspond to the intercept and slope of the line. The computed values of k_{23}, k_{32}, and K_{S_1} are listed in Table I. A calculated overall dissociation constant of 1×10^{-4} M agrees well with the thermodynamically measured constant of 2×10^{-4} M (Dahlquist, Jao and Raftery, 1966). Similar temperature jump data, obtained at pH 7.0 with both di-GlcNAc and tri-GlcNAc,

TABLE I

Rate and Equilibrium Constants for Lysozyme-Saccharide Binding

Temperature jump was from 29° to 38°. For other experimental conditions, see legend of Fig. 1. Constants refer to the mechanism:

$$E + S \overset{K_{S_1}}{\rightleftharpoons} ES_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ES_2$$

Sugar	pH	k_{23} (sec^{-1})	k_{32} (sec^{-1})	K_{S_1} (M)	Overall dissociation constant	
					Observed (M)	Calculated (M)
Di-GlcNAc	6.0	2×10^{4}	1×10^{3}	2×10^{-3}	2×10^{-4} [a]	1×10^{-4}
	7.0	1.5×10^{4}	2×10^{3}	1.5×10^{-3}		2×10^{-4}
Tri-GlcNAc	7.0	0.04×10^{4}	0.05×10^{3}	1.2×10^{-4}	1.6×10^{-5} [b]	1.5×10^{-5}

[a] Measured at 25° and pH 5.0 (Dahlquist, Jao and Raftery, 1966)
[b] Measured at 25° and pH 7.0 (Lehrer and Fasman, 1966)

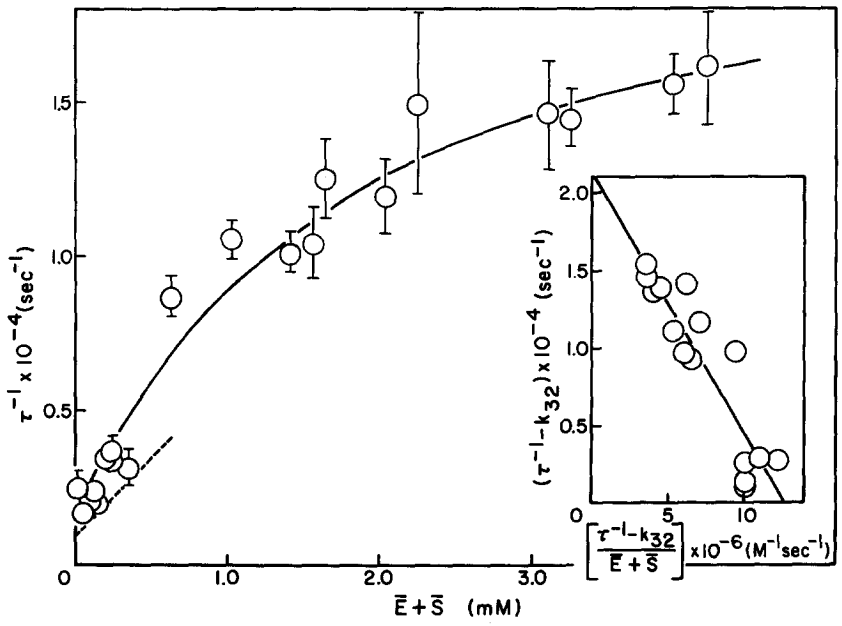

Fig. 1

The reciprocal relaxation time for lysozyme-di-GlcNAc complex formation at pH 6.0, as a function of the sum of the equilibrium concentrations of enzyme and substrate. Solid line, measurements following a temperature jump from 29° to 38°. Initial solution concentrations were 0.03 mM to 0.2 mM lysozyme (E), 0.02 mM to 4.1 mM di-GlcNAc (S), 0.6 mM p-nitrophenol, and 0.1 M Na_2SO_4. Values of equilibrium concentrations, $\bar{E}$ and $\bar{S}$, were computed with the use of the observed overall dissociation constants given in Table I. Each point represents the average of at least four determinations; error limits are the mean deviations of the determinations. The solid curve represents the line calculated from kinetic constants which were evaluated by a computer program based on Equation 3. A plot of the data according to Equation 3 is shown in the Fig. 1 inset, with the slope and intercept corresponding to K_{S_1} and k_{23}, respectively. The points in Fig. 1 inset represent the data shown in Fig. 1; the line was established by the computer program, which calculated k_{32}, by iteration, to give the best fit of the data to a straight line. The temperature jump apparatus has been described (Kresheek, Hamori, Davenport and Scheraga, 1966). The progress of the reaction was observed at 420 mμ with use of an interference filter obtained from Baird-Atomic, Inc.; λ_{max} is 420 mμ, with 42% transmittance and a 5 mμ half band width. The preparation of the saccharides has been described (Rupley, 1964). Comparative data of Chipman and Schimmel (1968), obtained at pH 6.0 following a temperature jump of 12° to 28.7°, are represented by the dashed line. In these experiments, initial solution concentrations were 0.02 mM to 0.08 mM lysozyme (E), 0.077 mM to 0.67 mM di-GlcNAc (S), 0.54 mM p-nitrophenol, and 0.1 M NaCl. It has been observed by Chipman and Schimmel (1968) that the indicator p-nitrophenol does not interact with lysozyme.

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are represented in Fig. 2. For these experiments also, the kinetic parameters (listed in Table I) were evaluated by use of Equation 3 and a computer

program. The solid line (for di-GlcNAc) and the dashed line (for tri-GlcNAc) were established by use of these kinetic parameters.

As can be seen in Fig. 1, the data at pH 6.0 (solid line) are in reasonable agreement with earlier temperature jump experiments of Chipman and Schimmel (1968), which are represented by the dashed line. These authors came to the conclusion that the binding of saccharide to lysozyme consists of a simple bimolecular process, even though the association rate constant was two orders of magnitude smaller than expected for a diffusion controlled process such as is usually observed as the first step in the binding of substrates to enzymes (Eigen and Hammes, 1963). It should be noticed from their data shown in Fig. 1 that Chipman and Schimmel did not use high enough reactant concentrations to be able to observe the concentration independence of τ.

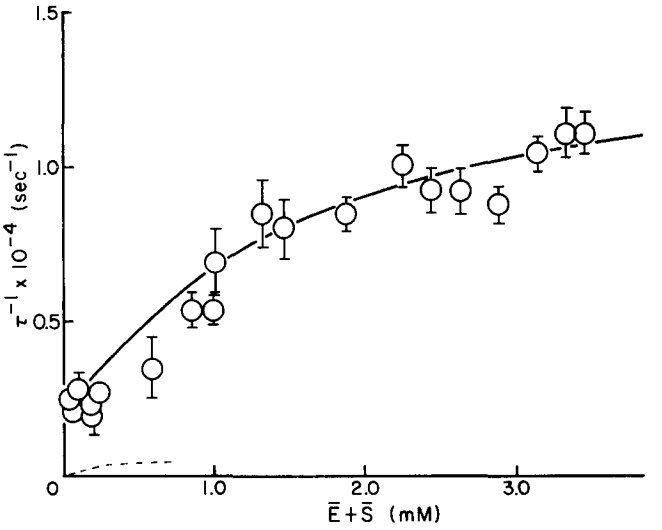

Fig. 2

The reciprocal relaxation time for lysozyme-saccharide complex formation at pH 7.0 as a function of the sum of the equilibrium concentrations of enzyme and substrate. Measurements were made following a temperature jump from 29° to 38°. Solid line, data for experiments with di-GlcNAc. Initial solution concentrations were 0.05 mM to 0.25 mM lysozyme (E), 0.05 mM to 4.5 mM di-GlcNAc (S), 0.2 mM p-nitrophenol, and 0.1 M Na_2SO_4. Each point represents the average of at least four determinations; error limits are the mean deviations of the determinations. The dashed line represents data obtained with tri-GlcNAc. In these experiments, initial solution concentrations were 0.05 mM to 2.0 mM lysozyme (E), 0.018 mM to 3.5 mM tri-GlcNAc (S), 0.5 mM p-nitrophenol, and 0.1 M Na_2SO_4. These curves represent the theoretical lines for the systems, calculated from the kinetic constants determined by use of Equation 3. For further details, see the legend of Fig. 1.

The mechanism shown in Equation 1, in which the diffusion controlled formation of a first enzyme-substrate complex is followed by a slower protein isomerization step, is consistent with the data in Figs. 1 and 2. Also, Sykes (1969) has reported that his NMR studies of the binding of methyl N-acetyl-D-glucosaminide to lysozyme are consistent with the mechanism of Equation 1. The second step shown in Equation 1 most probably represents a rearrangement of the protein as a result of substrate binding. This suggestion is consistent with data obtained from x-ray diffraction experiments of Phillips and co-workers (Blake, Johnson, Mair, North, Phillips and Sarma, 1967), which indicate that substrate binding to the A-C site is associated with a movement of tryptophan residue 62 of the enzyme. Substrate induced protein isomerization steps have previously been observed in ribonuclease-catalyzed reactions (French and Hammes, 1965), in chymotrypsin-catalyzed reactions (Moon, Sturtevant and Hess, 1965; Hess, 1968), and in reactions catalyzed by a number of other enzymes (Hammes, 1968). The time constants associated with this protein conformational change in lysozyme falls within the range (10^2 to 10^4 sec^{-1}) observed with other enzymes (Hammes, 1968).

It is to be noticed from the data in Table I that the specificity of the binding process expresses itself in the formation of both the first and the second enzyme-substrate complexes. The rate constants k_{23} and k_{32} for the protein isomerization step are 40 times faster for the dimer than for the trimer, presumably reflecting the fact that additional interactions occur with trimer. Clearly, both the formation of ES_1 and the protein isomerization allow for the enzyme to discriminate between these two sugars.

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