KINETICS OF LYSOZYME-SUBSTRATE INTERACTIONS[1]

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SUMMARY

Presteady state kinetics of the interaction of lysozyme with the hexamer and trimer of N-acetylglucosamine have been investigated by stopped-flow and relaxation methods. Two processes were observed with hexamer, and only one with trimer. The processes for hexamer were observed either as two consecutive proton uptake signals or as a fast increase followed by a slower decrease in fluorescence intensity. The faster of the two processes is identical to the process observed when trimer binds to the unproductive ABC sites of lysozyme. The slower of the two processes is presumably associated with the productive binding of hexamer with the enzyme.

Evidence has been presented (Rossi, Holler, Kumar, Rupley, and Hess, 1969) that the trimer and hexamer of N-acetylglucosamine bind in two main modes to lysozyme. The enzyme substrate dissociation constants pertaining to these binding modes have been determined. In view of crystallographic (Blake, Johnson, Mair, North, Phillips and Sarma, 1967; Blake, Mair, North, Phillips and Sarma, 1969) and chemical data (Rupley and Gates, 1967; Rupley, Butler, Gerring, Hartdegen and Pecararo, 1967) it has been suggested that these binding modes involve unproductive substrate binding to the ABC sites and productive binding to the ABC DEF sites of the enzyme. The kinetics of

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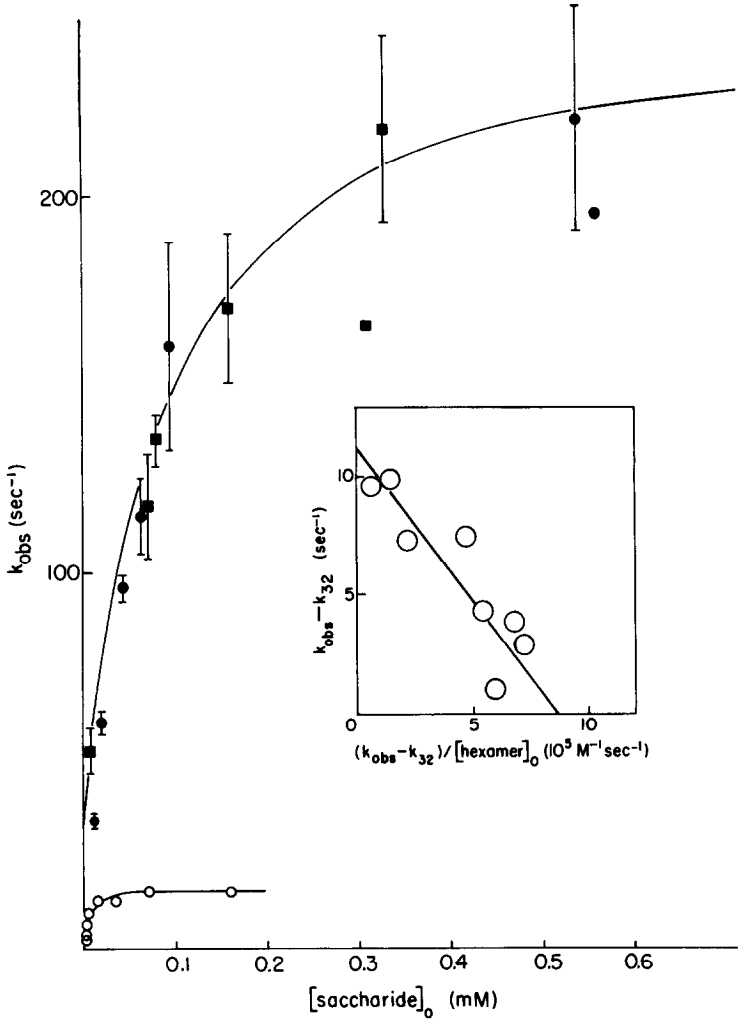

Fig. 1

The observed rate constant for the formation of the saccharide-lysozyme complex at pH 6.3, 20°C as a function of initial saccharide concentration. Upper solid line, stopped-flow experiments for trimer and hexamer (α-process) Each point represents the average of at least four determinations; error limits are the mean deviations. Initial concentrations were larger for the saccharides than for lysozyme (0.003 to 0.03 mM), except in the two single experiments where enzyme was in excess (k_{obs} is plotted versus enzyme concentration). Solutions contained 0,014 mM p-nitrophenol and 0.1 M Na_2SO_4. The filled circles, representing data for the trimer and the filled squares, representing data for the α-process of the hexamer follow the same concentration dependence. Lower solid line, measurements for hexamer (β -process). The solid lines were calculated from the kinetic constants in Table I which were evaluated by a computer program based on Equation 3. The points in Fig. 1 inset represent the data for the β-process shown in Fig. 1; the line was established by the computer program, which calculated k_{23} (intercept) and K_{S1} (slope) by iteration of k_{23} to give the best fit of the data to a straight line. The α-process was observed either as a proton uptake or an

increase in fluorescence intensity, and the β-process as either a proton uptake or a decrease in fluorescence intensity. In the experiments with indicator a Durrum-Gibson stopped-flow spectrophotometer was used at a wavelength set for maximum absorbance change. When the change in fluorescence intensity was observed the same stopped-flow apparatus was used but the light source had been exchanged by a high intensity Hg-Xe lamp. Scatter from the 295 nm excitation light was greatly reduced by use of the Durrum emission filter No. 2 with less than 1% transmittance at 300 nm. The temperature jump experiments have been described (Holler, Rupley and Hess, 1969). Saccharides were prepared according to Rupley, 1964.

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the binding of the dimer and trimer of N-acetylglucosamine to the ABC sites of the enzyme has been investigated using temperature-jump relaxation experiments (Holler, Rupley and Hess, 1969).

In this paper we report results for the interaction of the trimer and hexamer with lysozyme at pH 6.3. Measurements were performed by stopped-flow and temperature-jump relaxation methods. The course of the reaction was followed either by the color change of hydrogen indicators or by the fluorescence of the enzyme tryptophans (Lehrer and Fasman, 1966).

The following new observations are reported in this paper. The binding of trimer to lysozyme is characterized by a single fast relaxation indicated by a proton uptake or by an increase in fluorescence intensity. With hexamer a fast process (α-process) was observed identical with the relaxation for the trimer (Fig. 1). We found in addition a slower process (β-process), which is characterized by a proton uptake or by a decrease in fluorescence intensity. The reactions follow single exponential rate laws. The observed rate constants for both processes were found to obey a concentration dependence which is consistent with a two-step reaction scheme for each process

$$(1) \qquad E + S \underset{}{\overset{K_{S1}}{\rightleftharpoons}} ES_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ES_2 \qquad \begin{matrix}\alpha\text{-process} \\ \beta\text{-process}\end{matrix}$$

where the first step is in preequilibrium. When the saccharide concentration is in excess, the relation between the observed rate constant and the elementary kinetic constants is given by the equation

$$(2) \qquad k_{obs} = k_{32} + k_{23} \frac{[S]_o}{K_{S1} + [S]_o}$$

where $K_{S1} = [E] \cdot [S]_o / [ES_1]$. The equation may be written in linear form:

$$(3) \qquad k_{obs} - k_{32} = k_{23} - K_{S1} \cdot \frac{k_{obs} - k_{32}}{[S]_o}$$

The data for the β-process in Fig. 1 inset are plotted according to this relation. Evaluation of the kinetic constants has been described (Holler, Rupley and Hess, 1969). They are listed together with the calculated overall dissociation constants in Table I.

TABLE I

Rate and Equilibrium Constants for Lysozyme-Saccharide Binding at pH 6.3, 20°C

Constants refer to the scheme:

$$E + S \underset{}{\overset{K_{S1}}{\rightleftharpoons}} ES_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ES_2$$

Errors are given as standard deviations. For experimental conditions, see legend of Fig. 1.

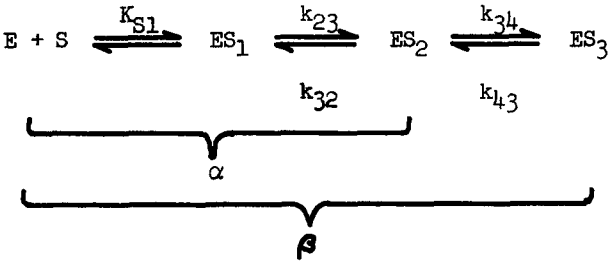
	k_{23} (sec^{-1})	k_{32} (sec^{-1})	K_{S1} (M) x10^5	Calculated overall dissociation constant (M) x 10^5
trimer and hexamer (α-process)	240±30	10±5	8±2	0.4±0.2
hexamer (β-process)	11±1	3.5±0.5	0.8±0.2	0.2±0.05

The calculated overall dissociation constant for the α-process of 4×10^{-6} M agrees well with the thermodynamically measured dissociation constant 6.6×10^{-6} M (pH 5.0, 25°C; Dahlquist, Jao and Raftery, 1966) for binding of trimer to sites ABC. We believe, that the β-process

reflects productive binding of hexamer to sites ABC DEF. The calculated overall dissociation constant 2 x 10^{-6} M for the β-process is in agreement with the dissociation constant of 5 x 10^{-6} M recently determined for productive binding of hexamer to the ABC DEF sites (Rossi, Holler, Kumar, Rupley and Hess, 1969).

It may be noticed from Fig. 1 that the β-process is observed after the α-process has approached equilibrium. The overall dissociation constant 4 x 10^{-6} M of the α-process is of the same magnitude as the dissociation constant 8 x 10^{-6} M for the preequilibrium step of the β-process Consequently, Equation 1 may be substituted by the following scheme:

(4)



Kinetic treatment of Scheme 4 results in expressions for k_{obs} which are experimentally indistinguishable from Equation 2. In contrast to Scheme 1 all complexes are productive in a sense that they lie on a single pathway. Whether Scheme 1 or Scheme 4 is correct is currently under investigation.

REFERENCES

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R., Proc. Roy. Soc., Ser. B, 167, 378 (1967).
Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R., Proc. Roy. Soc., Ser. B, 167, 365 (1967).
Dahlquist, F. W., Jao, L., and Raftery, M., Proc. Natl. Acad. Sci. U. S. 56, 26 (1966).
Holler, E., Rupley, J. A., and Hess, G. P., Biochem. Biophys. Res. Comm. 37, 423 (1966).
Lehrer, S. S., and Fasman, G. D., Biochem. Biophys. Res. Comm., 23, 133 (1966).
Rossi, G.-R., Holler, E., Kumar, S., Rupley, J. A., and Hess, G. P., Biochem. Biophys. Res. Comm., 37, 757 (1969).
Rupley, J. A., Biochim. Biophys. Acta, 83, 245 (1964).
Rupley, J. A., Butler, L., Gerring, M., Hartdegen, F. J., and Pecararo, R., Proc. Natl. Acad. Sci. U. S., 57, 1088 (1967).
Rupley, J. A., and Gates, V., Proc. Natl. Acad. Sci. U. S., 57, 496 (1967).