On the Mechanism of Lysozyme Catalysis : I. Hydrolysis of I-Benaoyl 2-Acetamido-2-Deoxy-β-D-Glucopyranoside

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https://doi.org/10.5109/22842

出版情報：九州大学大学院農学研究院紀要. 18 (1), pp.1-9, 1973-12. Kyushu University
バージョン：
権利関係：

I. Fac. Apr., Kyushu Univ., 18, 1-9 (1973)

On the Mechanism of Lysozyme Catalysis

I. Hydrolysis of 1-Benzoyl 2-Acetamido-2-Deoxy-β-D-Glucopyranoside

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(Received September 17, 1973)

1-O-Benzoyl 2-acetamido-2-deoxy-β-D-glucopyranoside was synthetized as a model compound of glycosyl-enzyme intermediate which may be considered as a possible intermediate in a lysozyme catalyzed reaction, The hydrolysis of this model compound by lynte species showed a distinctive plateau region in pH-rate profile. This may indicate that the model compound would be hydrolyzed through oxazoline intermediate which is formed by the participation of 2-acetamido group of the glucopyranose ring of substrate as a intramolecular nucleophile. The mechanism of the lysozyme catalysis was discussed on the basis of the experimental results.

INTRODUCTION

Lysozyme is one of enzymes of which the detailed catalytic mechanisms have been demonstrated. It has been well known that in a lysozyme catalyzed reaction, the protonated carboxyl group of Glu 35 acts first as a general acid for the cleavage of β-1,4-glycoside linkage, resulting in the C_1 carbonium ion intermediate at the reducing end of cleaved substrate, and second as a general base for the attack of water molecule or other nucleophiles on the C_1 carbonium ion intermediate, forming the reducing group or new glycoside linkages (Blake *et al.*, 1967). For occurring such catalytic reactions, the intermediate C_1 carbonium ion must be stabilized for a moment in some ways. For instance, the followings are thought to be possible mechanisms for stabilization of the intermediate : (1) The carbonium ion intermediate changes its structure to more stable one, (2) the charged intermediate interacts electrostatically with surrounding negative charges, (3) the intermediate is stablized by circumstantial conditions such as hydrophobic region (Hamaguchi and Hayashi, 1972).

There have been many proposals and discussions on the real intermediate structure which has been taken into the mechanism of lysozme catalysis. A glycosyl-lysozyme, which was thought to be formed by the reaction of the C_1 carbonium intermediate with carboxylate ion of Asp 52, was ruled out from the possible intermediate structure, because the distance between the C_1 carbonium ion and the carboxylate ion is too far to occur the reaction (Raftery and Rand-Meir, 1968). However, there are no other possible evidences to reject the gly-

cosyl-lysozyme as an intermediate structure. On the other hand, it was reported that some carbohydrolases contain carboxyl group or carboxylate ion as catalytic group, by which a glycosyl-enzyme would be formed as an intermediate of enzymatic reaction (Nishizawa and Hashimoto, 1970).

If the covalently bound glycosyl-enzyme were catalytically formed in an enzymatic reaction, this intermediate would also be catalytically decomposed to the product, because of its considerably high stability.

It is quite likely that the hydrolysis of esters of sugar and carboxylic acid in a mild condition was not studied in detail, though there were many data on the hydrolysis of the carboxylic esters such as sugar acetate in a drastic condition or in an organic solvent system.

It seems to be valuable to investigate the mechanism of hydrolysis of the sugar ester in an aqueous solution, presuming the most efficient pathway of the hydrolysis, since the information, thus obtained, could be used for a consideration of the glycosyl-enzyme intermediate. From the knowledge about the hydrolysis of a model compound, sugar ester, the mechanism of an enzyme catalysis may be easily established, if the saccharide substrate is hydrolyzed through the glycosyl-enzyme intermediate.

In this connection, 1-0-benzoyl 2-acetamido-2-deoxy-β-D-glucopyranoside was synthetized and hydrolyzed in aqueous solution. From the hydrolytic rate of the sugar ester, the possibility of glycosyl-lysozyme as intermediate in a lysozyme catalyzed reaction is discussed.

EXPERIMENTAL

Materials and methods

1-0-Benzoyl 2-acetamido-2-deoxy-4,6-O-benzylidene-β-D-glucopyranoside

2-Acetamido-2-deoxy-4,6-O-benzylidene-β-D-glucopyranose (8.6 g) synthetized according to Fletcher's method (Fletcher, 1963) was dissolved in **112** ml of absolute ethanol, and the solution was cooled to -5°C. One normal sodium methoxide (28 ml) was added to the solution. After standing the solution at -5°C for 15 min, the precipitated sodium salt was filtered, washed successively with absolute ethanol and ether, and dried under reduced pressure at room temperature. The dried sodium salt was suspended in 173 ml of dried dichloromethane and 6.8 ml of benzoyl chloride was added to the suspension. The suspension was shaken overnight at a room temperature, and then it was evaporated to dryness under reduced pressure. The dried material was dissolved in ***25*** ml of water and neutralized by saturated sodium bicarbonate solution using methyl red as an indicator. After decolorized with active carbon, the filtrate was cooled to 0°C for crystallization. The crystalline crop was recrystallized from ethanol, mp :185-187°C, $[\alpha]_D$= -45.1 (c=0.5, acetone).

1-0-Benzoyl 2-acetamido-2-deoxy-β-D-glucopyranoside (benzoyl NAG)

Palladium black **(0.5 g)** was suspended in ***60*** ml of methanol and saturated with hydrogen at the pressure of about 30 cm of water. Methanol was then decanted and a mixture of 3 g of 1-O-benzoyl 2-acetamido-2-deoxy-4,6-O-benzyli-

dene-β-D-glucopyranoside, *60* ml of methanol and 0.3 ml of glacial acetic acid was added. The reaction mixture was shaken with hydrogen gas until the adsorption of hydrogen has ceased (about 380 ml of hydrogen gas was consumed). After removing the catalyzer, the solution was evaporated under reduced pressure at 20°C to 50 ml. Five times volume of ether was added and the solution was kept in ice bath to complete the precipitation. The precipitate was crystallized from 6 parts of hot water, mp: 141-142°C, $[\alpha]_D = -34$ (c=1, water).

Kinetic measurement

Benzoyl NAG was dissolved in a buffer solution at desired pH value to the final concentration of 6.15×10^{-5} M, and the solution was incubated at 50°C in the cell of a Cary 14 spectrophotometer by circulating thermostatted water in the cell holder using a Haake type of water bath. The hydrolyzed amount was measured by difference spectrophotometry. Since benzoyl NAG shows a peak around *230* nm and benzoic acid around 240 nm in absorption spectra, a difference in the optical density, *A* OD, at 240-242 nm between the reaction mixture and aqueous solution of benzoyl NAG, which was stored in ice bath, was measured (see Fig. 1). The pseudo-first-order rate constant, k_{obs}, *of* the hydrolysis was obtained as a slope in the plotting of $\ln(\Delta OD_\infty/\Delta OD,)$ *vs* time *t*, where *A* OD, is the *A* OD value of reaction mixture hydrolyzed completely and *A* OD, the value at time *t* in second.

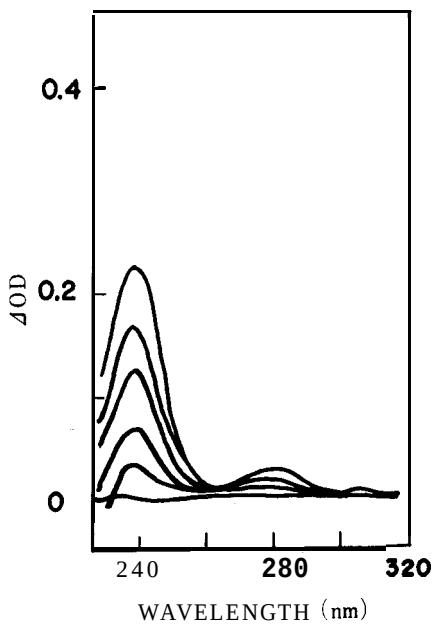


Fig. 1. The difference spectra of reaction mixture referred to solution of benzoyl NAG stored in ice bath.

RESULTS

Specific catalysis

The difference spectrophotometrically determined pseudo-first-order rate constant k_{obs} for the hydrolysis of benzoyl NAG at various pH values are listed in Table 1. The plotting of the k_{obs} ***vs*** pH value is shown in Fig. 2. The pH-log k_{obs} profile exhibits a characteristic plateau region from pH1 to 8.

Table 1. Kinetic constants for hydrolysis of benzoyl NAG.

Buffer	pH	a_H	K_w/a_H	k_{obs} ($\times 10^4$)
0.5 N HCl	0.3	0.5		2.18
0.1 N HCl	1.1	0.1		0.90
0.01 N HCl	1.8	1.58×10^{-2}		0.71
0.1 M acetate	4:o	1.00×10^{-4}		0.71
0.067 M phosphate	6.4	5.62×10^{-7}		0.77
0.067 M phosphate	8.0	$1.00 \times$ 10-R	6.7×10^{-6}	0.91
0.2 M carbonate	9.0	1.00×10^{-9}	6.7×10^{-5}	1.71
0.2 M carbonate	9.95	1.12×10^{-10}	5.98×10^{-4}	4.19
0.2 M carbonate	10.5	3.16×10^{-10}	2.12×10^{-3}	11.95

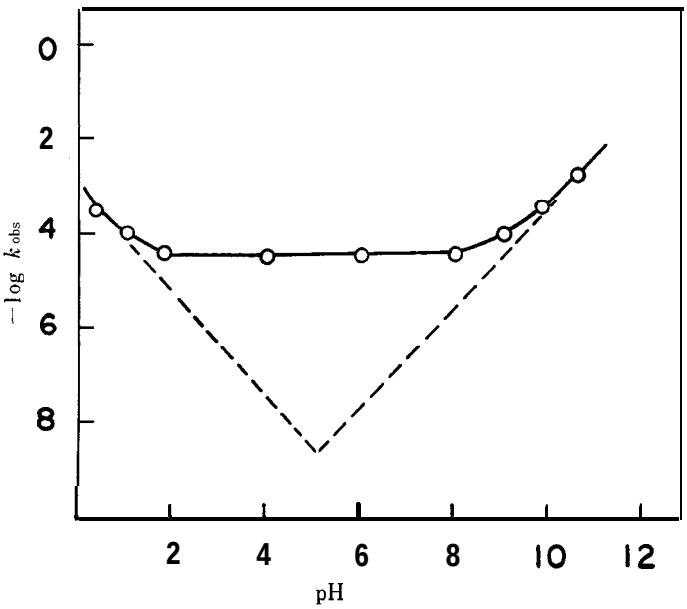


Fig. 2. Plotting of log k_{obs} *vs* pH.

The hydrolysis of benzoyl NAG by specific catalysis may follow a general rate equation :

$$k_{obs} = k_0 + k_H a_H + k_{OH} \frac{K_w}{a_H} \qquad (1)$$

where k_0 is the first-order rate constant for the spontaneous hydrolysis, k_H is

the secnd-order rate constant for the specific acid H^+ catalyzed hydrolysis, k_{OH} the second-order rate constant for the specific base OH- catalyzed hydrolysis and K_w the dissociation constant of water (6.7 x 10^{14} at 50°C). The equation (1) may be reduced to equations (2) and (3) according to the experimental conditions $a_H \gg K_w/a_H$ and $a_H \ll K_w/a_H$, respectively :

$$k_{obs} = k_O + k_H a_H \tag{2}$$

$$k_{obs} = k_O + k_{OH}\frac{K_w}{a_H} \tag{3}$$

The value of k_O may be estimated as the intercept in the plotting of k_{obs} *vs* a_H or K_w/a_H, and k_H and k_{OH} as slopes of the plotting, respectively as shown in Fig. 3.

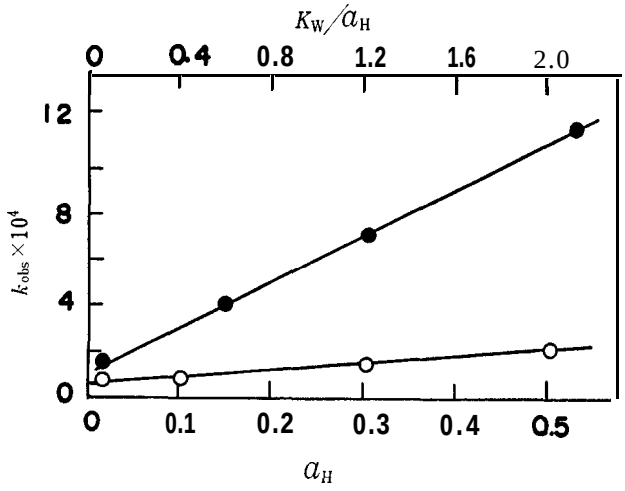


Fig. 3. Plotting of k_{obs} *vs* a_H and *vs* k_w/a_H. ○ : a_H, ● : k_w/a_H.

The value of rate constants, thus obtained, are listed in Table 2 together with those obtained for other glycosides (Piszkiewicz, 1967; Piszkiewicz and Bruice, 1968a). From the Table 2, several distinctive features of the hydrolysis of benzoyl NAG may be read; (1) the k_H value shows the same order as those of other glycosides, (2) the k_{OH} value of the ester is about 10^3 times larger than

Table 2. Kate constants of hydrolysis of various glycosides.*

Compound	$k_O(sec^{-1})$	$k_H(M^{-1}sec^{-1})$	$k_{OH}(M^{-1}sec^{-1})$
benzoyl NAG	0.70 x 10-4	2.95×10^{-4}	0.53
o-nitrophenyl β-NAG	1.45×10^{-4}	7.45×10^{-4}	6.4×10^{-3}
o-nitrophenyl a-NAG		3.72×10^{-4}	1.59×10^{-3}
p-nitrophenyl β-NAG	1.71×10^{-5}	2.97×10^{-4}	1.10×10^{-3}
p-nitrophenyl α-NAG		4.00×10^{-4}	0.95×10^{-3}
p-nitrophenyl /?-glucose	5×10^{-8}	1.38×10^{-4}	0.033
p-nitrophenyl a-glucose		6.20×10^{-4}	0.345

* Except for benzoyl NAG, hydrolysis was carried out at 78°C.

that of p-nitrophenyl glycoside of N-acetylglucosamine, (3) k_{III} value is 10^4 times larger than k_H value.

Acid-base catalysis

Imidazole

For the hydrolysis of benzoyl NAG, imidazole did not show any catalytic activity at various pHs. The same was true for acetic acid.

Tris(hydroxymethyl) aminomethane

Tris in base form exhibited a considerable catalytic effect for the hydrolysis of benzoyl NAG, whereas acid form did not show any effect as shown in Table 3. The hydrolysis of benzoyl NAG in basic Tris solution may be represented by following equation :

Table 3. Effect of Tris on hydrolysis of benzoyl NAG.

pH	concentration of Tris (M)	$k_{obs}(sec^{-1}) \times 10^{-4}$
6.7	0.081	0.804
6.7	0.405	0.784
7: 95	0.500	2.280
8.76	0.137	1.735
8.75	0.333	2.770
8.75	0.500	3.000
8.75	0.667	4.660

$$k_{obs} = k_O + k_H a_H + k_{OH}\frac{K_w}{a_H} + \left[k_b K_a/(a_H + K_a)\right], \quad v = k_{obs}(T)_o \qquad (4)$$

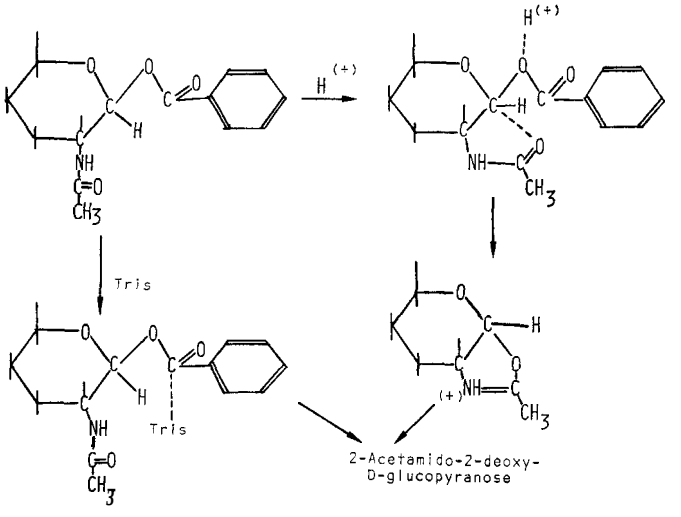
where k_b represents the second-order rate constant for catalytic hydrolysis by Tris in base form, K_a is the dissociation constant of Tris, $10^{-7.4}$, and (T), the total concentration of Tris. The slope of the plotting of k_{obs} *vs* $(T)_o$ gives the value of $k_b K_a/(a_H + K_a)$. The value of k_b was found to be $5.9 \times 10^{-4}\,M^{-1}sec^{-1}$, using the value of K_a and a_H obtained in specific acid catalysis.

DISCUSSION

The characteristic plateau region observable in the specific catalysis for the hydrolysis of benzoyl NAG may be arisen from the fact that this substance hydrolyzed through an oxazoline intermediate (see Scheme I). This assumption was supported by the observation that β-glycoside of 2-acetamido-2-deoxy-D-glucopyranose generally exhibited a plateau region in pH-hydrolytic rate profile, which was explained by the formation of 2-methyl Δ^2-(4,5-glucopyrano) oxazoline as an intermediate (Piszkiewicz and Bruice, 1967; Piszkiewicz and Bruice, 1968a ; Piszkiewicz and Bruice, 1968b). In contrary to β-glycoside, it has been well known that a-glycoside did not show the plateau region and has a great resistance for the hydrolysis in neutral region.

A large value of k_{OH} in the hydrolysis of benzoyl NAG may be caused by

Scheme I



the attack of hydroxide ion on the carbonyl carbon of ester linkage. If this is the case, the fission would occur at the bond between the oxygen atom and the carbonyl group of the ester linkage. Thus, after the hydrolysis, the oxygen atom of ester may be accommodated on C, atom of the sugar moiety.

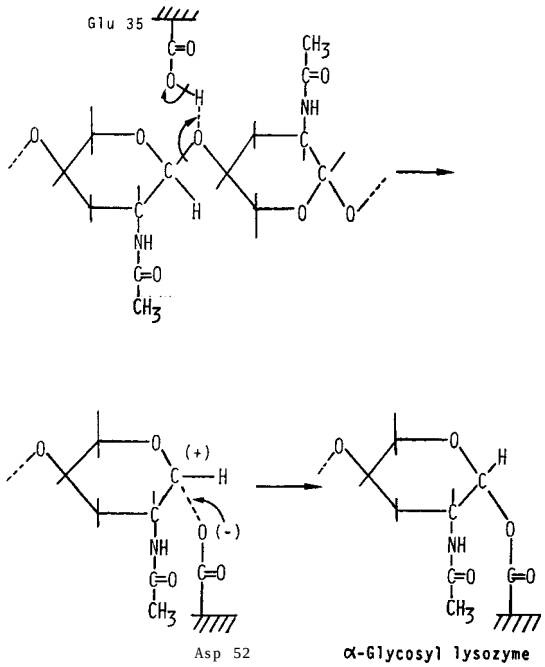
Acetic acid at pH *5.0,* imidazole around its p*K* value and Tris in protonated form did not exhibit any catalytic activity for the hydrolysis of benzoyl NAG. This may indicate that in test tube, benzoyl NAG was not able to hydrolyze by the general acid catalysis or the general base catalysis. Therefore, the catalytic activity of Tris in base form seemed to appear through its nucleophilic displacement on the carbonyl group of ester linkage, similar to the mode of action of specific base, OH^- (Bruice and York, 1961).

It has been reported that some saccharide-hydrolyzing enzymes contain specific carboxyl groups as the catalytic group. Carboxyl group as a catalyzer may act in three ways: that is, general acid catalyzer, general base catalyzer and nucleophilic catalyzer. However, at the present time, evidences that catalytic carboxyl group functions as nucleophilic catalyzer to result in the formation of covalently bound glycosyl-enzyme as in intermediate, have not yet been accumulated. The studies on the general possibility of glycosyl-enzyme as an intermediate (see Scheme II) in the hydrolysis or transglycosylation catalyzed by lysozyme may offer some information which may be used for judging the mode of action of catalytic carboxyl group in saccharide-hydrolyzing enzymes.

As shown by the present experimental results, generally sugar ester linkages seem to be more labile in the hydrolysis than ordinary glycoside linkages. This infers that the glycosyl-enzyme with ester linkage could be an intermediate of enzymatic hydrolysis, if an enzyme provided the structural requirement for occurring of rapid decomposition of such an intermediate.

There seems to be no concrete conclusion on the real role of Asp 52 carboxylate ion in a lysozyme catalyzed hydrolysis or transglycosylation. It was,

Scheme II



however, emphasized that carboxylate ion of Asp 52 is not able to form the ester linkage with C, carbonium ion, because both are too far distance to react easily (Blake *et al.*, 1967), if the sugar residue has been fixed at the subsite D invariantly during the reaction. Therefore, it is necessary to accumulate more evidences for ruling out the existence of glycosyl-enzyme as an intermediate in a lysozyme catalyzed reaction.

Let us assume that the glycosyl-lysozyme in P-form is the real intermediate. Then, this intermediate should be hydrolyzed or transglycosylated by the catalysis of general acid in the active site of the lysozyme molecule. In this case, the intermediate may convert to another intermediate sugar oxazoline. For the acceleration of the formation of the sugar oxazoline, general acid is thought to be most effective. Since in this step, Glu 35 was already changed to the conjugate base, there was no general acid in the active site of lysozyme. On the other hand, β-1,4-N-acetylglucosaminide linkage has been well known to be hydrolyzed by general acid to form directly the sugar oxazoline (Raftery and Rand-Meir, 1968). It may not be necessary to make such a detour *via* glycosyl-lysozyme for the formation of the sugar oxazoline.

Thus, it is concluded that the possibility of glycosyl-lysozyme as an intermediate in a lysozyme catalyzed reaction should be ruled out.

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