Use of Synthetic Substrates to Study Binding and Catalysis by Lysozyme*

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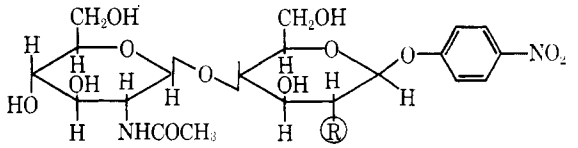
ABSTRACT: Three substrates for lysozyme have been synthesised by chemical and enzymatic methods and Michaelis–Menten parameters determined for their hydrolysis by the enzyme. They have the general structure



where R is acetamido (I), hydroxyl (II), or hydrogen (III). In addition, the cleavage pattern by the enzyme has been investigated in each case and it has been shown that for substrates II and III only the aryl glycosidic bond is cleaved. Substrate I has been shown to undergo cleavage at the aryl glycosidic bond to a lesser extent than at the other glycosidic bond. This finding makes substrate I unsuitable for determination of kinetic parameters. Substrate II has been used to investigate the effects of pH on K_m and V_{max}/K_m, and from these results pK_a values of amino acid side-chain-ionizable groups which are disrupted upon complex formation and which may be important in catalysis have been determined. Due to the small catalytic constant for hydrolysis of substrate II it has been possible to study its association with the enzyme by nuclear magnetic resonance methods. Results obtained have allowed definition of the region of the enzyme to which the substrate binds.

Kinetic studies of the mechanism of action of lysozyme have been hampered by the lack of substrates suited to this purpose. A major complication in this respect arises from the formation of nonproductive complexes involving three strong-binding subsites with small oligosaccharide substrates such as chitobiose or chitotriose, etc. If such inert complexes are competitive with others which undergo catalytic events any meaningful kinetic analysis is rendered difficult. Further complications arise from transglycosylation which occurs in addition to hydrolysis when oligosaccharide substrates are employed (Kravchenko, 1967; Dahlquist and Raftery, 1967; Rupley, 1967; Sharon, 1967). Several investigators (Osawa, 1966; Osawa and Nakazawa, 1966; Lowe, 1967; Lowe *et al.*, 1967; Lowe and Sheppard, 1968; Zehavi and Jeanloz, 1968; Raftery and Rand-Meir, 1968) have made use of aryl glycosides of chitobiose or chitotriose to study the kinetics of lysozyme action. In some of these investigations the findings that glycosidic bonds other than the aryl glycosidic one were cleaved by the enzyme and that transglycosylation also occurred were stressed while others did not take such complicating factors into account.

The investigation described here was initiated in the hope of finding a synthetic substrate in which a single glycosidic bond was cleaved by the enzyme and with which transglycosylation did not occur. Studies of such a substrate should afford meaningful kinetic data. In seeking such a substance we considered recent chemical evidence which has demonstrated that lysozyme: (1) binds chitin oligosaccharides to a series of subsites, three of which form a strong enzyme–inhibitor complex with chitotriose (Dahlquist *et al.*, 1966), (2) that specific magnetic parameters may be assigned to each of these three subsites (Raftery *et al.*, 1969a; Dahlquist and Raftery, 1969) and that such assignment allows definition of the relative modes in which many inhibitors occupy these sites, and (3) that the active (catalytic) site of the enzyme is occupied by Asp-52 (Parsons and Raftery, 1969) and by examining a model of lysozyme (Phillips, 1967; Blake *et al.*, 1967a) that this site is in a region of the molecule approximately 6 Å removed from the three strong-binding subsites. Furthermore, these chemical facts are in accord with the findings of X-ray analysis studies (Blake *et al.*, 1965; Phillips, 1967; Blake *et al.*, 1967a,b). On the basis of these latter investigations a proposal for the nature of the productive complex and for the catalytic mechanism has been advanced. It is suggested that this complex is an extension of the previously mentioned inert complex (subsites A, B, and C) through further weakly binding subsites (D, E, and F) and that catalytic events occur between subsites D and E through the intermediacy of two carboxyl side chains, acting as acid and base.

Since our studies of the binding of chitin oligomers to lysozyme (Raftery *et al.*, 1968; Dahlquist and Raftery, 1968a–c; Raftery *et al.*, 1969a) have shown that the three strong-binding subsites on the enzyme are occupied in the manner shown in Figure 1 it seemed reasonable to suppose that an oligosaccharide comprised of a NAG[1] residue (to bind in

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[1] Abbreviations used were: NAG-Glu-ϕNO_2, *p*-nitrophenyl 4-*O*-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-β-D-glucopyranoside; NAG-NAG-ϕNO_2, *p*-nitrophenyl 2-acetamido-4-*O*-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (*p*-nitrophenyl β-chitobioside); *p*-nitrophenyl-NAG, *p*-nitrophenyl 2-deoxy-2-acetamido-β-D-glucopyranoside; NAG-DG-ϕNO_2, *p*-nitrophenyl 4-*O*-(2-acetamido-

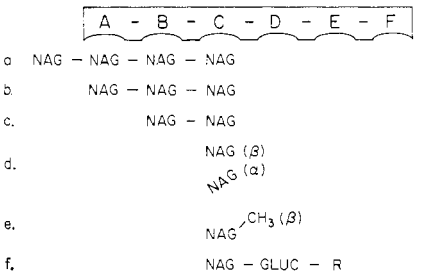

FIGURE 1: Scheme for relative binding modes of a series of oligosaccharides and glycosides to lysozyme. The enzyme is regarded as having a series of binding subsites (A–F) for 2-acetamidopyranose rings. Subsites A, B, and C form strong complexes with such rings while the other subsites do not. Reducing terminii of free saccharides occupy subsite C. In the glycoside NAG-Glu-R, R represents a methyl, phenyl, or *p*-nitrophenyl β-glycoside.

subsite C) linked to a hexose, such as glucose, which does not bind to sites A, B, or C, would fill sites C and D. As shown in Figure 1f, an aryl glycoside of this disaccharide might then be susceptible to hydrolysis by the enzyme since it would presumably traverse the catalytic site. To further reinforce this idea we have shown (Raftery and Rand-Meir, 1968) that glucosidic or 2-deoxyglucosidic bonds are susceptible to catalysis by the enzyme.

This communication describes the synthesis of such substrates for the enzyme and their use to delineate some of its kinetic properties.

Results

Synthesis of Oligosaccharides. A chemical synthesis of NAG-NAG-ϕNO_2 was carried out and was shown to yield material comparable with that described previously (Osawa, 1966). Enzymatic synthesis, utilizing the transglycosylase activity of lysozyme, was found to be a simpler route to disaccharide glycosides. A disaccharide glycoside prepared in this way (Figure 2) from chitotetraose, *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and lysozyme was shown to be identical with synthetic NAG-NAG-ϕNO_2 by analysis of its constituent parts and by paper chromatography, mixture melting point, ultraviolet, and proton magnetic resonance spectra. Similar enzymatic synthesis utilizing chitotetroase, lysozyme, and *p*-nitrophenyl β-D-glucopyranoside led to isolation (Figure 3) of the disaccharide NAG-Glu-ϕNO_2. The glycosidic bond formed by transglycosylation was shown to be 1–4 by oxidation with periodate followed by reduction with borohydride, hydrolysis, and determination of the glycerol and erythritol formed. Each mole of the disaccharide consumed 3 moles of periodate which showed that the mode of linkage was either 1–4 or 1–2. The finding that equimolar amounts of glycerol and erythritol were formed following oxidation, reduction, and hydrolysis confirmed the linkage as 1–4. The stereochemistry of the enzymatically synthesized bond was considered to be exclusively of the β configuration due to the finding (Dahlquist *et al.*, 1969) that retention of

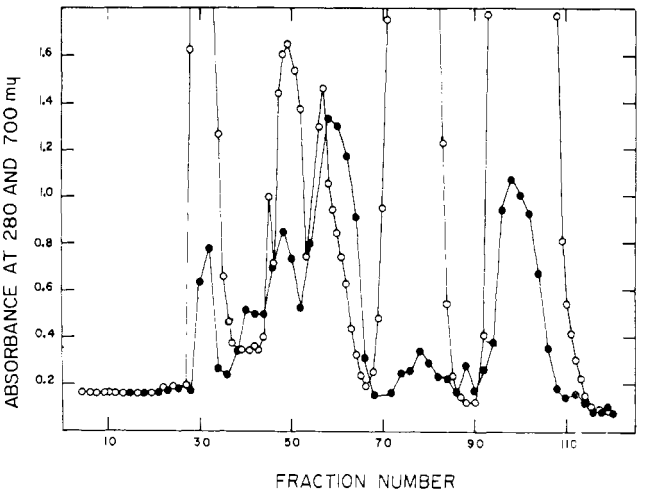

FIGURE 2: Chromatography of a mixture containing lysozyme, chitotetraose, and *p*-nitrophenyl-NAG following incubation at 40° for 16 hr. Absorbance at 280 mμ due to *p*-nitrophenyl glycosides is shown as open circles. Absorbance at 700 mμ due to reducing groups (Park–Johnson, 1949, method) is shown as closed circles. Fractions 28–33 contained lysozyme, 70–85 contained NAG-NAG-ϕNO_2, and 90–110 contained *p*-nitrophenyl-NAG.

configuration obtains to an extent of at least 99.7% in hen egg-white lysozyme catalyzed transglycosylation. A disaccharide, NAG-DG-ϕNO_2, was synthesized enzymatically in a similar fashion using *p*-nitrophenyl 2-deoxy-β-D-glucopyranoside as acceptor. The configuration of the glycosidic bond was assumed to be β.

It is well known that a detailed kinetic analysis of the action of lysozyme on most substrates is complicated by strong catalytically nonproductive binding which competes with the weaker productive binding of the substrate. Under these conditions, the observed value of K_m reflects the nonproductive rather than the productive association of the substrate and enzyme. The observed V_{max} is related to the actual catalytic step by the ratio of the nonproductive to productive

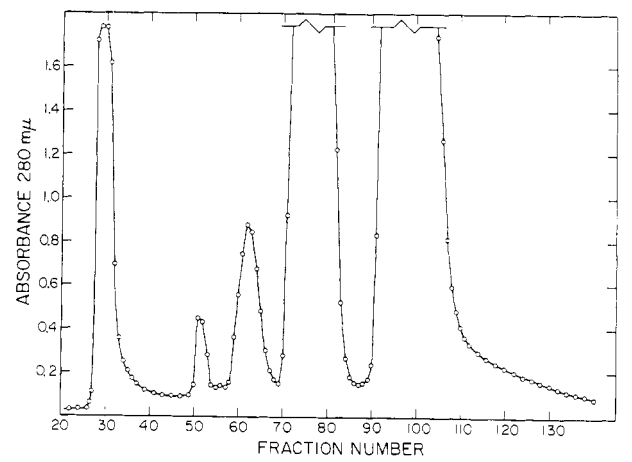

FIGURE 3: Chromatographic fractionation of the incubation mixture of lysozyme, chitotetraose, and *p*-nitrophenyl β-D-glucoside. Fractions 26–35 contained lysozyme, 58–67 contained NAG-NAG-Glu-ϕNO_2, 70–83 contained NAG-Glu-ϕNO_2, and 90–110 contained Glu-ϕNO_2. Details are given in the Experimental Section.

2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside; NAG-Glu-ϕ, phenyl 4-*O*-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-β-D-glucopyranoside.

TABLE I: Michaelis–Menten Parameters for Lysozyme-Catalyzed Hydrolysis of Synthetic Substrates.

	[S] $\times 10^{-2}$ M	[E] $\times 10^{-4}$ M	V_{max} (moles/sec)	K_m (M)	V_{max}/K_m (sec^{-1})
NAG-Glu-ϕNO_2	0.56–3.35	1.8	$(1.7 \times 0.1) \times 10^{-9}$	$(2.8 \pm 0.8) \times 10^{-9}$	7.3×10^{-8}
NAG-NAG-ϕNO_2	0.52–1.55	1.8	$(1.5 \pm 1.0) \times 10^{-8}$	$(2.9 \pm 0.4) \times 10^{-8}$	5.2×10^{-7}
NAG-DG-ϕNO_2	0.54–1.62	1.8	$(2.2 \pm 0.5) \times 10^{-8}$	$(8 \pm 6) \times 10^{-8}$	3×10^{-6}

binding constants. However, the value V_{max}/K_m gives the ratio of the catalytic rate constant to the value of K_m' for the catalytically important binding mode. We shall therefore consider K_m, V_{max}, and V_{max}/K_m as the important kinetic parameters.

The values of K_m, V_{max}, and V_{max}/K_m for the lysozyme catalyzed hydrolysis of the three synthetic substrates NAG-NAG-ϕNO_2, NAG-Glu-ϕNO_2, and NAG-DG-ϕNO_2 are shown in Table I. The determined K_m was found to be generally the same for all three substrates and to be about equal to previously determined dissociation constants, K_s, for α-NAG or β-NAG or the α- and β-methyl glycosides of NAG (Raftery *et al.*, 1968, 1969a,b). On the other hand, the catalytic constants which were calculated for NAG-NAG-ϕNO_2 and NAG-Glu-ϕNO_2 differed from each other by one order of magnitude. While the study presented here was in progress a preliminary report of similar work has appeared (Lowe and Sheppard, 1968) describing differences in these constants for the same two substrates. These authors have presented an argument suggesting that the greater catalytic constant obtained for NAG-NAG-ϕNO_2 is due to anchimeric assistance by the acetamido side chain at C_2 of the sugar ring undergoing enzymatic glycosyl rupture. These workers have invoked the idea that NAG-Glu-ϕNO_2 binds productively to the enzyme in sites C–D–E whereas the strongest mode of binding of NAG-NAG-ϕNO_2 is in sites B–C–D (nonproductive) and that productive binding of this substrate is much weaker. Consequently the observed difference in V_{max} for the two substrates is smaller than the true difference.

Before making a comparison of the determined kinetic parameters we have investigated the products of lysozyme action on all three synthetic substrates under a variety of conditions. A simple method for product analysis involved the use of gel filtration columns to separate the enzyme, glycosides, and free sugars (Raftery *et al.*, 1969b). By such means it was shown that NAG-Glu-ϕNO_2, NAG-Glu-ϕ, and NAG-DG-ϕNO_2 were cleaved specifically by the enzyme to give the corresponding disaccharide and the free aglycone. No cleavage of the glycosidic bond distal from the aglycone was found. In addition, no transglycosylation occurred to form higher molecular weight oligosaccharides or oligosaccharide glycosides. A representative chromatogram is shown in Figure 4. In contrast to this clear specificity of bond cleav-

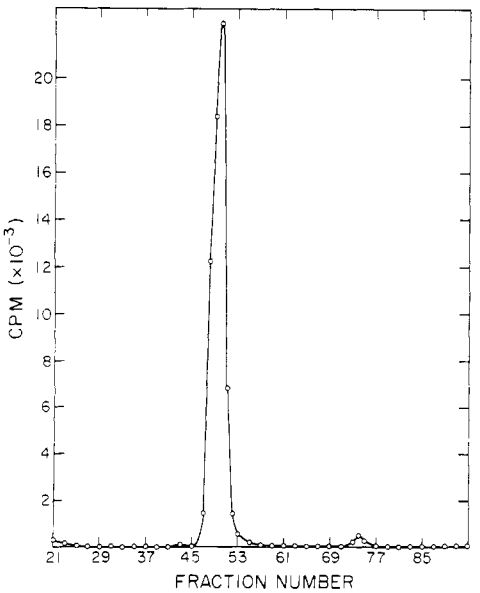

FIGURE 4: Chromatography on a column of Bio-Gel P-2 of the hydrolysis mixture obtained from incubation of lysozyme and NAG-Glu-ϕ-^{3}H for 18 hr at 40°, pH 5.5. The peak at tube 49 represents NAG-Glu-ϕ-^{3}H while that at tube 74 represents phenol-^{3}H. No Glu-ϕ-^{3}H was obtained.

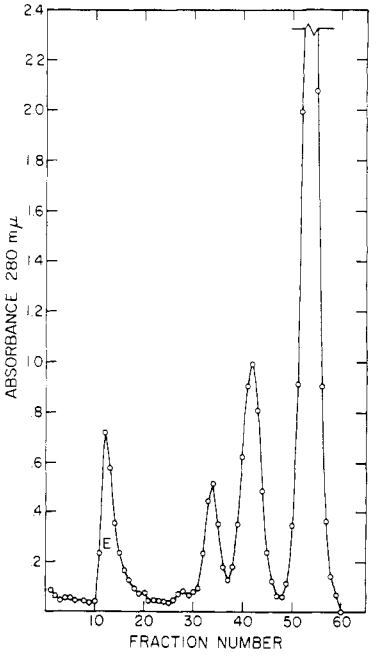

FIGURE 5: Chromatography of a mixture of lysozyme and NAG-NAG-ϕNO_2 (E:S = 1:40) after 16-hr incubation at 40°. E refers to the fractions containing lysozyme. Fractions 32–37 contained NAG-NAG-NAG-ϕNO_2, 38–46 contained NAG-NAG-ϕNO_2, and 49–59 contained NAG-ϕNO_2. Details of the chromatography are given in the Experimental Section of the text.

TABLE II: Relative Amounts of Cleavage of the Two Glycosidic Bonds of NAG-NAG-ϕNO_2.

E:S	% *p*-NO_2-phenol[a]	% NAG-ϕNO_2[a]	Incubn Time (hr)
10:1	13	5	20
1.5:1	15	20	21
1:40	3	60	16

[a] The percentage of the original substrate NAG-NAG-ϕNO_2 released as *p*-nitrophenol or *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside.

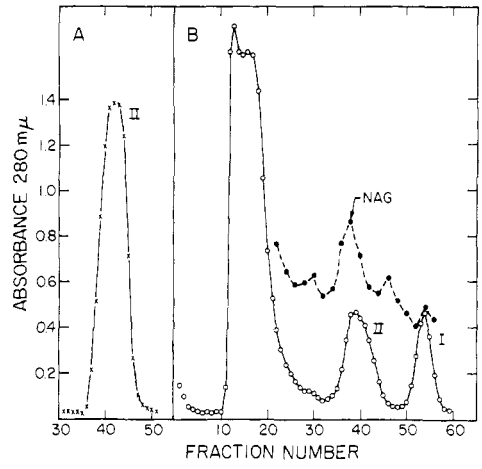

FIGURE 6: Chromatography studies. (A) Of NAG-NAG-ϕNO_2 on a Bio-Gel P-2 column. Crosses denote absorbance at 280 mμ. (B) Of an incubation mixture (21 hr, 40°) of lysozyme and NAG-NAG-ϕNO_2 (E:S = 1.5:1.0). Open circles denote absorbance at 280 mμ; closed circles denote reducing groups determined by the Park–Johnson method.

age it was found that under conditions of $E_0 > S_0$ or $E_0 < S_0$ the cleavage pattern of NAG-NAG-ϕNO_2 by lysozyme was extremely complex. Under conditions similar to those used to show a clean cleavage of the aryl glycosidic bond of NAG-Glu-ϕNO_2 the product distribution shown in Figure 5 was obtained using NAG-NAG-ϕNO_2 as substrate. Clearly extensive cleavage of the NAG–NAG glycosidic bond had occurred to yield large amounts of NAG-ϕNO_2 and transglycosylation had also occurred yielding *p*-nitrophenyl glycosides of oligosaccharides higher than chitobiose. A comparison of the amounts of the residual NAG-NAG-ϕNO_2 with the *p*-nitrophenol produced (determined before application to the column) showed the per cent cleavage of the bonds A and B ($NAG\overset{A}{-}NAG\overset{B}{-}\phi NO_2$) to be 60 and 3%, respectively. Similar results were obtained using $E_0 > S_0$ and $E_0 \gg S_0$ as shown in Figures 5 and 6. These results are presented in Table II.

Since complicating factors such as transglycosylation or multiple bond cleavage were not involved in the lysozyme-catalyzed hydrolysis of NAG-Glu-ϕNO_2 this substrate was utilized in an investigation of the effects of pH on the catalytic properties of lysozyme. Figure 7B and A shows such effects on K_m and V_{max}/K_m, respectively. The results demonstrate that one ionizable group on the enzyme is perturbed by its strongest association with NAG-Glu-ϕNO_2. This group has an apparent pK_a in the free enzyme of approximately 5.6–5.8 which is changed to a value of 6.1–6.3 upon association with the substrate. The plot of log V_{max}/K_m *vs.* pH shows that an ionizable group with a pK_a in this same region affects either the catalytic rate or productive binding and it is reasonable to assume that the same amino acid side chain is involved in both cases. In addition, at least two other groups of lower pK_a have an effect on the catalytic rate or productive binding. The data are not sufficiently accurate to assign a definite value to these groups except to state that they likely have pK_a values in the region of 2–4.5. The importance of such ionizable groups will be dwelt upon in the Discussion section.

The design of a substrate such as NAG-Glu-ϕNO_2 was based on the notion that the NAG moiety would bind to the strongest binding subsite, subsite C (Raftery *et al.*, 1969a), and this would place the glucose residue in subsite D with the aryl glycosidic bond being subject to catalysis according to the scheme of Blake *et al.* (1967b) for productive binding. Our recent use of nuclear magnetic resonance to study enzyme–inhibitor association (Raftery *et al.*, 1968, 1969a; Dahlquist and Raftery, 1968a–c) has provided us with a ready means of discerning whether inhibitor molecules bind to subsite C in lysozyme since specific magnetic parameters have been assigned to it. Due to the slow catalytic rate of lysozyme-catalyzed hydrolysis of NAG-Glu-ϕNO_2 we were able to study its association with the enzyme, employing our nuclear magnetic resonance method. It was found that the NAG moiety of the substrate does indeed bind to subsite C, as evidenced by the observed chemical shift of its acetamido methyl resonance to higher field in the presence of the enzyme (Table III). The dissociation constant obtained (Table III) by this method ($K_s = 1.5 \pm 0.3 \times 10^{-2}$ M) agrees quite well with the value of K_m ($2.3 \pm 0.8 \times 10^{-2}$ M) obtained from the kinetic analysis of substrate hydrolysis. These results suggest that the productive mode of binding is at least *competitive with* if not identical with binding in subsites C, D, and E of NAG-Glu-ϕNO_2.

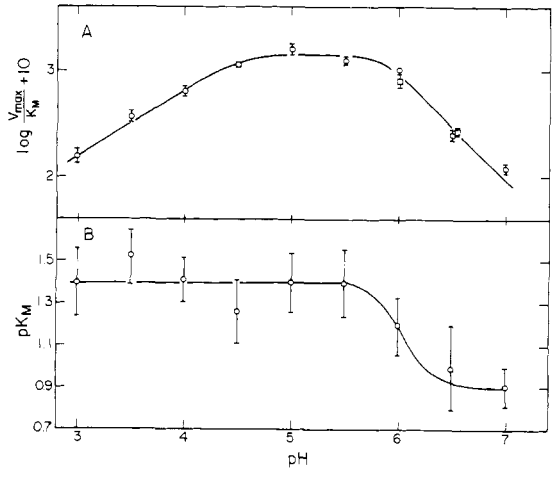

FIGURE 7: Plot of $-\log K_m$ *vs.* pH for lysozyme-catalyzed hydrolysis of NAG-Glu-ϕNO_2.

TABLE III: Data for the Binding of Substrates to Lysozyme As Determined by Nuclear Magnetic Resonance Methods.

	K_s	Δ (ppm)[a]
NAG-ϕNO_2	$3.4 \pm 0.3 \times 10^{-2}$	0.37 ± 0.03
NAG-Glu-ϕNO_2	$1.5 \pm 0.3 \times 10^{-2}$	0.43 ± 0.03
NAG-NAG-ϕNO_2[b]		$0.50 - 0.60$

[a] Chemical shifts for enzyme-bound acetamido methyl groups. [b] The chemical shift reported is for the acetamido methyl group proximal to the nitrophenyl group.

The association of NAG-NAG-ϕNO_2 with lysozyme has also been studied by the nuclear magnetic resonance method. It was seen that at pH 5.5, 40°, the acetamido methyl group proximal to the aglycone underwent a large chemical shift to higher field (Table III) upon association with the enzyme while the distal acetamido methyl group was found to be shifted only slightly in the same direction. This means that the preferred mode of binding of this substrate is in subsites B and C, with the nitrophenyl moiety probably in site D. The strength of its binding is not in the range of the determined K_m ($2.9 \pm 0.4 \times 10^{-2}$ M) since the plots of the chemical shift data showed that the dissociation constant was probably smaller than 10^{-3} M. These results mean either that (1) productive binding of NAG-NAG-ϕNO_2 is not competitive with its strong mode of association with subsites B, C, and D or (2) the kinetic data are unreliable due to the complex manner of hydrolysis of this compound.

Discussion

The finding that the lysozyme-catalyzed cleavage of NAG-NAG-ϕNO_2 yields nitrophenol as the minor product and NAG-ϕNO_2 as the major one, under widely varying conditions of enzyme and substrate concentrations, casts serious doubt on the validity of any kinetic parameters determined for this substrate on the basis of nitrophenol release. In view of the observed transglycosylation it is possible that a higher molecular weight derivative is the source of nitrophenol, such a substrate being present in some steady-state concentration. A similar multiple cleavage pattern has been observed in the lysozyme-catalyzed cleavage of benzyl 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (Zehavi and Jeanloz, 1968). Presumably the multiple cleavage of all of these aryl chitobiosides is due to multiple modes of association with a series of binding subsites on the enzyme surface. Due to this complexity, and especially since release of the aglycone is the minor pathway, numbers obtained from the enzymatic release of nitrophenol from NAG-NAG-ϕNO_2 *cannot* be interpreted as true Michaelis–Menten parameters. We therefore believe that the data of Lowe and Sheppard (1968) should not be used to support the notion of a mechanism which involves anchimeric assistance by the acetamido side chain of the substrate.

The binding of NAG-NAG-ϕNO_2 to the enzyme as determined by nuclear magnetic resonance methods (Raftery *et al.*, 1968, 1969a,b; Dahlquist and Raftery, 1968a–c) showed that this substance binds predominantly in subsites B, C, and D; *i.e.*, nonproductively. It is possible that it also binds productively *or* nonproductively in subsites C, D, and E although it is certain that it binds in other ways which lead to catalysis; *e.g.*, in subsites D, E, and F. Because of the demonstrated differences in binding modes of NAG-NAG-ϕNO_2 and NAG-Glu-ϕNO_2 and the serious differences in cleavage patterns of both substrates it is our opinion that the kinetic data are not sufficient to be regarded as proof of anchimeric assistance in 2-acetamido group containing substrates. This does not mean, of course, that such a mechanism may not obtain for such substrates.

In contrast to the complications observed while using NAG-NAG-ϕNO_2 as a substrate the use of NAG-Glu-ϕNO_2 gave rather clear-cut results. The nuclear magnetic resonance data showed that it binds with its NAG moiety in subsite C as evidenced by the chemical shift to higher field of its acetamido methyl group due to complexation (Raftery *et al.*, 1969a,b). Therefore the rest of the molecule should occupy subsites D and E. The agreement between the determined K_s and K_m shows that this binding in subsites C, D, and E is *competitive with* if not actually identical with the productive mode of binding. Further agreement in this respect is seen by comparing the present results of plotting pK_m *vs.* pH for NAG-Glu-ϕNO_2 with previous results from this laboratory on the binding of methyl-β-NAG to lysozyme (Dahlquist and Raftery, 1968b). In that study, by nuclear magnetic resonance methods, the effects of pH were observed on pK_s and on the chemical shift of the acetamido methyl group of the inhibitor. In the nuclear magnetic resonance study it was observed that a single ionizable group, $pK_a = 6.1$ on the enzyme, $pK_a = 6.6$ on the complex, affected the magnitude of the dissociation constant. In the present instance, using NAG-Glu-ϕNO_2 a single ionizable group was also observed; the pK_a value, although not determined with the same accuracy, was $pK_a \sim 5.8$ on the enzyme changing to $pK_a \sim 6.3$ on the complex. The difference in the determined values in the two studies is undoubtedly due to the presence of 10% (v/v) dioxane in the buffers used in the present work. This was included to help solubilize the substrate at concentrations greater than 10^{-2} M. Calculations based on the data of Findlay *et al.* (1962) for the effects of organic solvents on the properties of ribonuclease A show that the effects observed here are in accord with their reasoning. In our work we used citrate buffers, *i.e.*, neutral acids, and the ionizable group on the enzyme has been shown previously to be a carboxyl group (Blake *et al.*, 1967a; Dahlquist and Raftery, 1968b), most likely Glu-35. The raising of both pK_a values, for the buffer and for the carboxyl on the enzyme, as a result of the added dioxane would have a net effect of zero on the pH binding curve of the substrate. The finding, however, that the curve was slightly shifted to lower pH argues that the result expected for the organic–aqueous buffer was obtained but that no change in pK_a of the carboxyl group on the enzyme results from adding 10% dioxane to the medium. This is not unexpected since glutamic-35 is in a solvent-*inaccessible* environment (Blake *et al.*, 1967a,b) in the native enzyme, a condition which accounts for its high pK_a value. Therefore our previous work on the binding of methyl-β-NAG and the present results on the Michaelis parameters for NAG-Glu-ϕNO_2 complement each other and provide reasonable evidence that the substrate occupies subsites C, D, and E.

It is especially interesting that such conclusions have been reached since the active site of lysozyme has been postulated to be between subsites D and E from crystallographic evidence (Blake *et al.*, 1967b; Phillips, 1967).

In addition, we have shown (Parsons and Raftery, 1969) that chemical modification of a single carboxyl on the enzyme, Asp-52, which lies in the region postulated for the active site, leads to complete inactivation of lysozyme. Proof that Asp-52 plays an actual role in catalysis is, however, difficult to obtain. To date there is no knowledge of the chemical properties of Asp-52. Preliminary results (S. M. Parsons and M. A. Raftery, unpublished data, 1969) suggest that in the free enzyme it has a pK_a of approximately 3.5. In this regard it is therefore of interest that the plot of log V_{max}/K_m *vs.* pH in the present study shows a dependence upon ionizable groups having p K_a values in the region of 2–4.5 (Figure 7A). The slope of the curve above pH 6 is -1 and represents the effect of one ionizable group on the parameter V_{max}/K_m. However, the apparent slope in the region 3–4.5 is about 0.6. Most probably this is the result of two effects working in opposition. One possible explanation involves a group of pK_a $\sim$ 2 which is perturbed to pK_a $\sim$ 4.5 by productive binding. Another group of pK_a $\sim$ 3.5 could be required for catalysis in its basic form. Thus, two groups, one acting as an acid, the other as a base, interact to give the apparent slope of 0.6. A theoretical curve for such a situation is not, however, described by a straight line. Realistically it cannot be said that these results implicate Asp-52 in any mechanism (Raftery and Rand-Meir, 1968) for the following reason. The catalytic constant for hydrolysis of NAG-Glu-ϕNO_2 is low and this could mean that the strongest mode of binding in subsites C, D, and E does not have the precise orientation necessary for catalysis but that a slightly different (energetically unfavorable) orientation in the same subsites does lead to catalysis. This differentiation could be affected by pH in the sense that Asp-52 might cause small changes in the local structure of the enzyme depending upon its ionization state, one of which (the ionized form) is more favorable for binding which leads to catalysis. In general, care should be exercised in the interpretation of plots of log V_{max}/K_m *vs.* pH since separation of orientation effects (perhaps due to conformation changes) and catalytic effects is difficult if not impossible.

In the lysozyme-catalyzed hydrolysis of NAG-Glu-aryl substrates additional information is available which allows *considerable definition* of the mechanism. We have shown (Dahlquist *et al.*, 1968) that the transition state for such substrates involves considerable carbonium ion character. It is reasonable to conclude in addition from X-ray analysis (Blake *et al.*, 1967a,b), nuclear magnetic resonance data (Raftery *et al.*, 1969; Dahlquist and Raftery, 1968b), and protein chemical information (S. M. Parsons and M. A. Raftery, 1969, unpublished data) that at the active site of the enzyme two carboxylic acids, Asp-52 and Glu-35, occur with pK_a values of approximately 3.5 and 6.1, respectively. The profile for catalysis reflects these pK_a's and it is reasonable to tentatively suggest that Glu-35 is acting as a general acid, with protonation of a glycosidic oxygen. It is more difficult to suggest a specific role for Asp-52. It could function by formation of an ion pair with the developing carbonium ion in the transition state but it should be emphasized that there is no evidence for such a function. Whereas a considerable number of the details of lysozyme-catalyzed cleavage of glycosidic bonds have now been elucidated there are still many vexing questions about several aspects of the mechanism.

Experimental Section

Materials and Methods. Lysozyme (Lot no. 668-8590) was purchased from Sigma Chemical Co. *p*-Nitrophenyl β-D-glucoside was purchased from Cyclo Chem. Co. 2-Deoxyglucose was obtained from Pierce Chemical Co. and *N*-acetyl-D-glucosamine was obtained from Calbiochem. Chitobiose and chitotetraose were obtained by acid hydrolysis of chitin followed by isolation by gel filtration methods (Raftery *et al.*, 1969a).

Reducing sugars were estimated by the Park–Johnson method (Park and Johnson, 1949). *p*-Nitrophenol concentrations were determined by dilution of the samples in 0.1 M potassium tetraborate (pH 9.2) and measurement of optical densities at 400 mμ, employing a molar extinction coefficient of 18,000. *p*-Nitrophenyl glucosides were determined by measurement of optical densities at 280 or 265 mμ. *N*-Acetyl-D-glucosamine was determined by the Morgan–Elson (1934) method. Optical density measurements were made using either a Cary Model 14 or a Gilford Model 240 spectrophotometer. Nuclear magnetic resonance spectra were recorded using either a Varian Model A60-A or Varian Model HA-100 spectrometer. The Michaelis–Menten parameters K_m and V_{max} were obtained by use of the equation

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{s} + \frac{1}{V_{max}}$$

employing Lineweaver–Burk plots. An Olivetti-Underwood Programma 101 desk computer was used to calculate the K_m and V_{max} parameters and to estimate the errors by the least-squares method.

Preparation and Isolation of p-Nitrophenyl 4-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside. Lysozyme (0.5 g), chitotetraose (0.5 g), and *p*-nitrophenyl β-D-glucopyranoside (1.5 g) were dissolved in 100 ml of citrate buffer (0.1 M, pH 5.2) and 10 ml of dioxane and a few drops of toluene were added. The mixture was incubated at 40° for 16 hr and then passed through a column (4 × 200 cm) of Bio-Gel P-2, using 0.1 M NaCl as eluting solvent. Fractions (24 ml) were collected. The absorbance of each fraction was read at 280 mμ to determine the presence of *p*-nitrophenyl glucosides. Aliquots (20 μl) from every other fraction were analyzed for reducing sugars by the Park–Johnson (1949) method. The tubes corresponding to the various fractions were pooled and treated with Amberlite MB-1 to effect desalting and removal of reducing sugars. The yield was 150 mg (NAG-Glu-ϕNO_2), mp 256–258° (uncor).

Characterization of NAG-Glu-ϕNO_2. NAG-Glu-ϕNO_2 (5 mg) was dissolved in 10 ml of water. A 2-ml aliquot was hydrolyzed, after dilution with 2 ml of 3 N HCl at 100° for 30 min. This solution (labeled A) was used for determination of the glucose to *p*-nitrophenol ratio. Glucose was estimated by adding 3.0 ml of the orcinol reagent to 1.0 ml of solution A, heating to 100° for 10 min, and comparing the optical density at 500 mμ with that of a standard solution of glucose. *Anal.* Calcd concentration of glucose in A: 4.95×10^{-4} M.

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Anal. Calcd concentration of *p*-nitrophenol: 1.68×10^{-2} M. Found: 1.61×10^{-3} M.

Determination of the Michaelis–Menten Parameters. A. HYDROLYSIS OF NAG-Glu-ϕNO_2. Varying concentrations of substrate [$(0.5\text{–}3.4) \times 10^{-2}$ M] were incubated with a constant concentration of enzyme (3.6 or 1.8×10^{-4} M) in a final volume of 1.0 ml of citrate buffer (0.1 M, pH 5.0) which contained 10% v/v dioxane. Blank solutions contained the same concentrations of the substrate but no enzyme. Samples of 200 μl were taken at intervals and diluted with 3.0 ml of potassium tetraborate, and the concentration of *p*-nitrophenol released with time was determined. K_m and V_{max} were derived from plots of $1/s$ *vs.* $1/v$.

B. HYDROLYSIS OF NAG-NAG-ϕNO_2. The parameters for this substrate were obtained by the same method as for NAG-Glu-ϕNO_2. The substrate concentrations were $(0.5\text{–}1.48) \times 10^{-2}$ M and the enzyme concentration was 1.8×10^{-4} M. The solvent used contained 15% (v/v) dioxane.

C. HYDROLYSIS OF NAG-Glu-ϕNO_2. The parameters were obtained by the same method used for NAG-Glu-ϕNO_2. The substrate concentrations were $(0.54\text{–}1.62) \times 10^{-2}$ M and the enzyme concentration was 1.8×10^{-4} M.

pH Dependence of K_m and V_{max} for the Hydrolysis of NAG-Glu-ϕNO_2. Varying concentrations of substrate [$(0.75\text{–}2.5) \times 10^{-2}$ M] were incubated at 40° with a constant concentration of enzyme (3.6×10^{-4} M) in a final volume of citrate buffer (0.1 M, pH 3.0–7.0) which contained 10% (v/v) dioxane for 24 hr. Blank solutions contained the same concentrations of the substrate but no enzyme. The whole samples were then diluted with 2.0 ml of potassium tetraborate and filtered through a Millipore filter, and the *p*-nitrophenol concentrations were estimated by measuring the optical density at 400 mμ. The Michaelis–Menten parameters were obtained by plotting $1/s$ *vs.* $1/v$.

Specificity of Bond Cleavage by Lysozyme. A. HYDROLYSIS OF NAG-NAG-ϕNO_2. (i) Using Excess Substrate (E:S = 1:40). Lysozyme (5 mg) and the substrate (7.5 mg) were dissolved in citrate buffer (pH 5.0; 0.1 M containing 15% dioxane v/v) and incubated for 16 hr at 40°. The reaction mixture was applied to a Bio-Gel P-2 column (0.9×70 cm); 1.25-ml fractions were collected and the absorbance of each at 280 mμ was determined. (ii) Using Excess Enzyme (E:S = 1.5:1). Lysozyme (50 mg/ml) and substrate (1.1 mg/ml) were incubated in a final volume of 2 ml of citrate buffer (pH 5.0; 0.1 M + 15% dioxane) for 21 hr at 40°. An aliquot of 0.3 ml was diluted with 3.0 ml of 0.1 M borate and read at 400 mμ (optical density 0.475), yield of *p*-nitrophenol = 15%; 0.82 ml was applied on a small Bio-Gel P-2 column (0.9×70 cm) and eluted with 0.1 M NaCl. Fractions of 1.25 ml were collected, and read at 280 mμ. Aliquots of 50 μl were taken from every other fraction and analyzed for reducing sugars by the Park–Johnson (1949) method. An aliquot of 0.82 ml of the original solution (before incubation) was chromatographed on a similar column, and fractions of the same size were collected and their absorbancies were read at 280 mμ.

B. HYDROLYSIS OF NAG-Glu-ϕ. The synthesis of [^{3}H]phenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside (NAG-Glu-ϕ) was carried out using conditions similar to those employed for the synthesis of NAG-NAG-ϕNO_2 and is described elsewhere (Dahlquist *et al.*, 1969). The specific activity of this compound was found to be 0.5 mCi/mmole. The lysozyme-catalyzed hydrolysis of NAG-Glu-ϕ was performed at 40° in 0.1 M citrate buffer (pH 5.8) with a drop of toluene to inhibit bacterial growth. The concentration of substrate used was 10^{-2} M while the enzyme concentration was 3×10^{-3}. After incubation for 18 hr, an aliquot of the reaction mixture was applied to a 0.9×70 cm column of Bio-Gel P-2 (200–400 mesh) and eluted with 0.1 M sodium chloride. An aliquot of (100 μg) each of the 1.0-ml fractions was added to 15 ml of Bray's solution (Bray, 1960) and the ^{3}H activity was determined on a Packard Tri-Carb liquid scintillation spectrometer, Model 3324.

The dissociation constants of lysozyme with NAG-ϕNO_2, NAG-NAG-ϕNO_2, and NAG-Glu-ϕNO_2 were determined by proton magnetic resonance methods (Raftery *et al.*, 1968, 1969a; Dahlquist and Raftery, 1968a–c). These measurements were performed with a Varian HA 100 nuclear magnetic resonance spectrometer. Solutions were made to contain 3×10^{-3} lysozyme in 85% 0.1 M citrate buffer (pH 5.0) and 15% dioxane. Substrate or inhibitor concentrations ranged from 7 to 50 mM.

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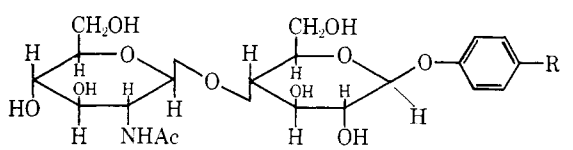
Application of Secondary α-Deuterium Kinetic Isotope Effects to Studies of Enzyme Catalysis. Glycoside Hydrolysis by Lysozyme and β-Glucosidase*

F. W. Dahlquist, T. Rand-Meir, and M. A. Raftery

ABSTRACT: Secondary kinetic isotope effects (α-deuterium) have been explored as a method for delineating the nature of the transition state in enzyme-catalyzed reactions. Hen egg-white lysozyme and almond β-glucosidase were chosen as enzymes for these experiments. Initially, model studies were performed on the cleavage of phenyl glucosidic bonds in acid (SN1 mechanism) and in base (SN2 mechanism). The values obtained (k_H/k_D = 1.13 for SN1 mechanism, k_H/k_D = 1.03 for SN2 mechanism) were in the anticipated range. The value obtained for β-glucosidase-catalyzed hydrolysis of phenyl β-D-glucopyranoside was k_H/k_D = 1.01, and this suggests a displacement mechanism for this enzyme. Such a result is in agreement with recent chemical evidence. For application of the method to studies of the lysozyme mechanism, the disaccharide phenyl 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-β-D-glucopyranoside was synthesized by enzymatic transglycosylation using ^{3}H- and ^{14}C-labeled phenyl glucosides (C_1-H and C_1-*d*, respectively). The value of k_H/k_D obtained for the lysozyme-catalyzed hydrolysis of this aryl disaccharide glycoside was determined to be 1.11. This result indicates considerable carbonium ion character in the transition state of the enzyme-catalyzed reaction. It was found that this result held over the pH range 3.1–8.3 as well as close to the pH optimum.

The X-ray analysis studies of Blake *et al.* (1965, 1967a,b) on the binding of various inhibitors and substrates to lysozyme has led to a mechanistic proposal for catalysis (Blake *et al.*, 1967a; Phillips, 1967). This involves catalytic production of a carbonium ion which is given steric and electrostatic stabilization by the enzyme. Recent chemical studies have shown that (a) lysozyme-catalyzed hydrolysis of a glycosidic bond proceeds with retention of configuration to at least 99.7% (Dahlquist *et al.*, 1969); (b) aryl *glucosides* of the type



serve as substrates for the enzyme (Raftery and Rand-Meir, 1968; Rand-Meir *et al.*, 1969) with exclusive cleavage of the aryl glucosidic bond; (c) for such glucosidic substrates the mechanism of hydrolysis involves a carbonium ion intermediate (Dahlquist *et al.*, 1968). Evidence for such an intermediate was obtained by use of secondary α-deuterium kinetic isotope effects to study the enzyme-catalyzed reaction. This communication describes further kinetic isotope effect studies on model glucosides as well as extension of the lysozyme studies to ensure uniformity of mechanism over a wide range of pH. Application of the kinetic isotope method to study the mechanism of β-glucosidase-catalyzed hydrolysis of aryl glucosides is also described.

Experimental Section

All pH measurements were performed with a Radiometer pH Meter 26. The ultraviolet spectrophotometric measurements were carried out with a Gilford 240 spectrophotometer. Proton magnetic resonance spectra were obtained with a

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