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EFFECT OF GLYCOSIDIC ISOLOGS IN LYSOZYME CATALYSIS

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SUMMARY

Glycosidic isologs of *p*-methylphenyl-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside in which the aryl glycosidic oxygen is substituted by sulfur or selenium, have been synthesized. No substantial difference is observed for the isologs in their abilities to form nonproductive complexes with lysozyme (aminopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17). However, the isologs differ in rates and patterns of hydrolysis and transglycosylation. Relative reactivities of the isologs for the enzymic hydrolysis follow the order (O ≥ S > Se) of the acid-catalyzed hydrolysis of model compounds. This is in agreement with the proposed carbonium ion mechanism for lysozyme catalysis.

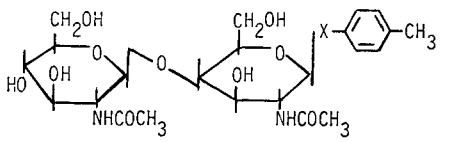
INTRODUCTION

Hen's egg-white lysozyme (aminopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) has been the subject of numerous investigations[1,2]. In particular, its primary, secondary and tertiary structures have been elucidated[3–5]. From X-ray crystallographic studies of lysozyme–glycose complexes, BLAKE *et al.*[6] suggest that the lysozyme-catalyzed hydrolysis of chitooligoses proceeds by general acid catalysis *via* an intermediary carbonium ion which is stabilized by the enzyme. Other mechanisms, such as general acid catalysis assisted anchimerically by the acetamide group[7] and general acid-nucleophile catalysis[8], have been considered. Transglycosylation greatly complicates the mechanistic study of lysozyme-catalyzed hydrolysis[9,10], because it leads to complex reaction mixtures and products, and causes peculiarities in the kinetics of hydrolysis. Therefore, information concerning the mechanism of lysozyme catalysis can best be obtained at this stage by studying the effect of substrate structures on the rate of hydrolysis rather than a detailed kinetic analysis.

In view of the position of sulfur and selenium in the periodic table and their

* The following abbreviations are used throughout: NAG, 2-acetamido-2-deoxy-*D*-glucopyranose or *N*-acetylglucosamine; NAG_2, chitobiose; NAG_3, chitotriose; NAG_4, chitotetraose; MO-NAG 1-*O*-*p*-methyl-phenyl-2-acetamido-2-deoxy-β-D-glucopyranoside; MS-NAG, 1-*S*-*p*-methylphenyl-2-acetamido-2-deoxy-β-D-thioglucopyranodide; MSe-NAG, 1-*Se*-*p*-methylphenyl-2-acetamido-2-deoxy-β-D-selenoglucopyranoside; $MO\text{-}NAG_2$, 1-*O*-β-*p*-methylphenyl chitobioside $MS\text{-}NAG_2$, 1-*S*-β-*p*-methylphenyl thiochitobioside; $MSe\text{-}NAG_2$, 1-*Se*-β-*p*-methylphenyl selenochitobioside.

electronic and steric effects relative to those of oxygen[11], sulfur and selenium containing isologs of oxygen compounds have been studied in chemical and enzymic systems[12,13]. The establishment of the mechanism of acid-catalyzed hydrolysis of β-glucopyranosides has been aided by use of the corresponding thio compounds[14,15]. Sulfur and selenium isologs of *p*-methylphenyl-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)2-2deoxy-β-D-glucopyranoside were synthesized in order to compare their reactivities toward lysozyme. Results are discussed in terms of isolog effects on lysozyme catalysis.



X = O : MO-NAG$_2$

S : MS-NAG$_2$

Se : MSe-NAG$_2$

EXPERIMENTAL

Chemicals

Twice crystallized, salt-free lysozyme from hen's egg-white was obtained from Worthington Biochemical Corp. Chitin was the product of Sigma Chemical Co. *N*-Acetylchitooligoses were prepared by a partial hydrolysis of chitin with HCl[16,17]. 2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glycopyranose (NAG tetraacetate) was purchased from Pierce Chemical Co., and 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,3,6-tri-*O*-acetyl-2-deoxy-β-D-gluco-pyranose (chitobiose octaacetate) was prepared as described previously[18,19]. *p*-Cresol and *p*-thiocresol were purchased from Eastman Organic Chemicals. *p*-Selenocresol was synthesized by reacting selenium and *p*-methylphenyl magnesium bromide which was prepared from *p*-bromotoluene[20]. The product was purified by vacuum distillation and the fraction with b.p. 40° (1.4 mm Hg) was collected. The compound had m.p. 45.5–46.0°. Silica gel G was obtained from Research Specialties Co.

Synthesis of 1-O-p-methylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (MO-NAG) and its isologs

The condensation of NAG tetraacetate with *p*-cresol in the presence of *p*-toluene sulfonic acid at 100° *in vacuo* (25 mm Hg)[21] gave *p*-methylphenyl-2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranoside which was *O*-deacetylated with 1 M sodium methoxide to MO-NAG. Recrystallization of MO-NAG from aqueous methanol gave the product, m.p. 247° (decomp.). The NMR spectrum ([$^2H_6$]dimethylsulfoxide showed δ at 1.83 (s, acetamido CH_3), 2.24 (s, *p*-aryl CH_3) and 7.00 ppm (q, aryl H). (Found: C, 5.2; H, 6.7; N, 4.1. $C_{15}H_{21}O_6N \cdot H_2O$ requires C, 54.7; H, 7.0; N, 4.3%).

An identical procedure was employed to synthesize 1-*S*-*p*-methylphenyl-2-acetamido-2-deoxy-β-D-thioglucopyranoside (MS-NAG) and 1-*Se*-*p*-methylphenyl-2-acetamido-2-deoxy-β-D-selenoglucopyranoside (MSe-NAG). Recrystallization of MS-

NAG from aqueous methanol gave the product, m.p. 246–247° (decomp.). The NMR spectrum ([$^2H_6$]dimethylsulfoxide) showed δ at 1.83 (s, acetamido CH_3), 2.26 (s, *p*-aryl CH_3) and 7.24 ppm (q, aryl H). (Found: C, 54.8; H, 6.7; N, 4.3; S, 9.6. $C_{15}H_{21}O_5NS$ requires C, 55.0; H, 6.5; N, 4.3; S, 9.8%).

Recrystallization of MSe-NAG from water gave the product, m.p. 247–248° (decomp.). The NMR spectrum ([$^2H_6$]dimethylsulfoxide) showed δ at 1.83 (s, acetamido CH_3), 2.26 (s, *p*-aryl CH_3) and 7.26 ppm (q, aryl H). (Found: C, 48.3; H, 5.6; N, 3.6; Se, 21.3. $C_{15}H_{21}O_5NSe$ requires C, 48.2; H, 5.3; N, 3.7; Se, 21.1%).

Synthesis of p-methylphenyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (MO-NAG$_2$) and its isologs

Chitobiose octaacetate was converted into 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (α-acetochlorochitobiose) by the method of OSAWA[22]. The condensation of α-acetochlorochitobiose with *p*-cresol in alkaline acetone yielded MO-NAG$_2$ pentaacetate which was O-deacetylated with 1 M sodium methoxide to give MO-NAG$_2$ (ref. 19). Recrystallization from water gave white needles, m.p. 307–309° (decomp.), $[\alpha]^{24}_D = -15.3$ (c 0.4 in acetic acid).

An identical procedure was employed to synthesize 1-*S*-*p*-methylphenyl-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)2-deoxy-β-D-thioglucopyranoside (MS-NAG$_2$) from *p*-thiocresol and 1-*Se*-*p*-methylphenyl-2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-selenoglucopyranoside (MSe-NAG$_2$) from *p*-selenocresol. Recrystallization of MS-NAG$_2$ from water gave white needles, m.p. 296–297° (decomp.), $[\alpha]^{24}_D = -18.0$ (c 0.25 in acetic acid). The infrared spectrum (KBr) showed absorption (cm^{-1}) at 3300 (OH, NH), 1655, 1550 ($NHCOCH_3$), 1490 (aryl C=C) and 885 (β-glycoside). The NMR spectrum ([$^2H_6$]dimethylsulfoxide) showed δ at 1.84 (s, acetamido CH_3), 2.26 (s, *p*-aryl CH_3) and 7.25 ppm (q, aryl H). (Found: C, 49.6; H, 6.1; N, 5.1; S, 5.9. $C_{23}H_{34}N_2O_{10}S \cdot H_2O$ requires C, 50.4; H, 6.6; N, 5.1; S, 5.8%).

Recrystallization of MSe-NAG$_2$ from water gave white needles, m.p. 270–271° (decomp.), $[\alpha]^{24}_D = -23.2$ (c 0.28 in acetic acid). The infrared spectrum (KBr) showed absorption (cm^{-1}) at 3300 (OH, NH), 1655, 1550 ($NHCOCH_3$), 1490 (aryl C=C) and 885 (β-glycoside). The NMR spectrum ([$^2H_6$]dimethylsulfoxide) showed δ at 1.83 (s, acetamido CH_3), 2.27 (s, *p*-aryl CH_3) and 7.26 ppm (q, aryl H). (Found: C, 46.8; H, 5.9; N, 4.9; Se, 13.6. $C_{23}H_{34}N_2O_{10} \cdot SeH_2O$ requires C, 46.4; H, 6.1; N, 4.7; Se, 13.3%).

Analytical methods

Melting points were obtained by means of a Fisher–Jones hot stage apparatus. Optical rotations were determined using a Perkin–Elmer polarimeter, model 141 and pH measurements were made using a Radiometer TTTIc. Infrared spectra were obtained by use of a Perkin–Elmer grating infrared spectrophotometer, Model 225 and NMR spectra were taken by means of JEOLCO JNM-60 and Varian T-60 instruments. Difference ultraviolet spectra at a constant lysozyme concentration ($1.24 \cdot 10^5$ M) and varied substrate concentrations (10^{-3} to 10^{-5} M) were taken in a Cary 14 spectrophotometer using a 0–0.1 absorbance slide wire assembly at room temper-

ature[23]. The elementary analyses were performed by Pascher Mikroanalytisches Laboratorium, Bonn, West Germany.

Studies of lysozyme catalysis

Lysozyme-catalyzed hydrolysis of MO-NAG_2, MS-NAG_2 and MSe-NAG_2 were carried out as previously described[19]. The liberation of *p*-cresol, *p*-thiocresol and *p*-selenocresol was followed spectrophotometrically at 279, 265 and 244 nm, respectively. Lysozyme-catalyzed transglycosylation was studied by product analyses using thin-layer chromatography. Thin layer plates (20 cm × 20 cm) were coated with Silica Gel G in 0.02 M citrate buffer (pH 5.1). The lysozyme digest (5 ml) was treated with chloroform, centrifuged and lyophilized to a white powder which was dissolved in 1.0 ml of water. An aliquot of 10 μl was applied to the plate which was developed with *n*-propanol–ethanol–water (70:96:34, by vol.). The plate was sprayed with 50% H_2SO_4 in methanol and heated at 130° for 10 h. The plates were analyzed by scanning in a spectrodensitometer (Model SD 3000, Schoeffel Instrument Corp.) equipped with Honeywell Electronik 19 recorder and SDC 300 density computer at 450 nm. Standard curves were used to convert areas under the curves into concentrations.

RESULTS

Lysozyme interacts with *N*-acetylchitooligoses to form nonproductive complexes which exhibit a red shift in ultraviolet spectra. The difference spectra for the interaction of lysozyme with MO-NAG_2, MS-NAG_2 and MSe-NAG_2 are shown in Fig. 1. The maximum difference at 293.5 nm is attributed to the hydrophobic pertur-

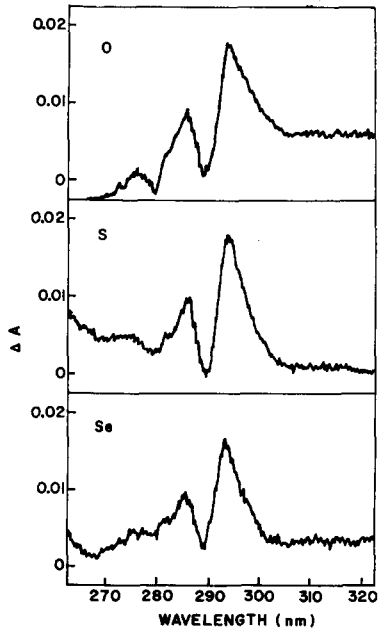
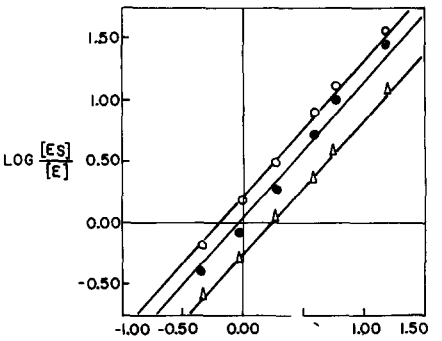


Fig. 1. Difference ultraviolet spectra of lysozyme ($1.24 \cdot 10^{-5}$ M) in the presence of $1 \cdot 10^{-3}$ M of MO-NAG_2, MS-NAG_2 and MSe-NAG_2.



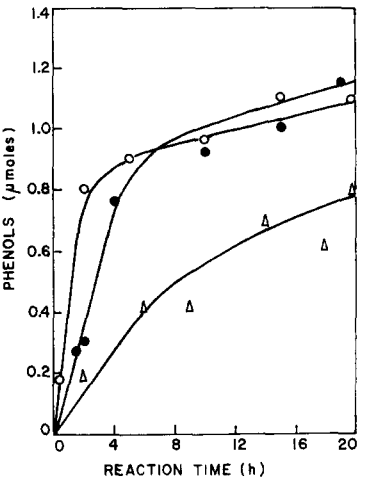


Fig. 2. Graphical determination of dissociation constants of MO-NAG_2 (○), MS-NAG_2 (●) and MSe-NAG_2 (△) from difference spectral data.

Fig. 3. Rates of lysozyme-catalyzed hydrolysis of MO-NAG_2 (○), MS-NAG_2 (●) and MSe-NAG_2 (△). Reaction mixtures containing 0.05 mM of lysozyme and 2.5 mM of substrates in 0.1 M citrate buffer (pH 5.1) were incubated at 45°. Samples were withdrawn at suitable time intervals for the determination of phenols by spectrophotometry.

TABLE I

BINDING AND HYDROLYSIS OF SYNTHETIC SUBSTRATES BY LYSOZYME

Substrate	*Dissociation constants (K_s) for nonproductive complexes (M) × 10^4*	*Initial velocities (v) of phenol liberation ($\mu moles \cdot h^{-1}$) × 10^3*
MO-NAG_2	0.84 ± 0.15	3.6 ± 0.9
MS-NAG_2	0.71 ± 0.15	2.2 ± 0.6
MSe-NAG_2	1.78 ± 0.35	0.5 ± 0.2

TABLE II

CHEMICAL HYDROLYSIS OF 2-ACETAMIDO-2-DEOXY-β-D-GLYCOPYRANOSIDE AND ITS ISOLOGS

Reaction mixtures which contain $0.2 \cdot 10^{-4}$–$2 \cdot 10^{-4}$ M of MO-NAG, MS-NAG or MSe-NAG in 1 M HCl or 1 M NaOH (I = 0.3 in KCl) were incubated at 45°. Aliquots of 1.0 ml were withdrawn at intervals for the analysis of phenol liberated. Rates were corrected for the solvolysis.

	k_{H^+} (h^{-1}) × 10^4	k_{OH^-} (h^{-1}) × 10^4
MO-NAG	20.5	0.1
MS-NAG	1.9	0.6
MSe-NAG	1.5	3.8

bation of tryptophan residue of the enzyme by substrates[24], and the variation in its peak height with substrate concentrations can be used to estimate $[ES]/[E]$ (ref. 25). Plots of log $[ES]/[E]$ *vs.* log $[S]$ as shown in Fig. 2 give straight lines with intercepts corresponding to $-\log K_s$. Slopes, being equal to one, are in accord with the formation of 1:1 nonproductive complexes between the enzyme and substrates. Since the glycosidic oxygen is not implicated in the interaction of nonproductive complexes, one would not expect a large difference in dissociation constants for the isologous compounds. An apparently high dissociation constant for MSe-NAG_2 may be due to experimental uncertainties caused by the insolubility of the seleno compound.

Fig. 3 shows that all three isologous compounds are hydrolyzed by lysozyme. Due to the complexicity of their reactions, no attempt was made to calculate rate constants. Initial velocities for the liberation of phenols correlate with the rate order of acid-catalyzed hydrolyses (Table II).

Lysozyme is known to catalyze transglycosylation in addition to hydrolysis[25,9]. *N*-Acetylchitooligoses has been separated into their components by chromatographic methods and thin-layer chromatography was found to be particularly useful for the product analyses of lysozyme digests. Fig. 4 shows scanning records of lysozyme digests of MO-NAG_2, MS-NAG_2 and MSe-NAG_2. Two components, A and B are visualized under ultraviolet light. Component A is identical to synthetic MO-NAG or

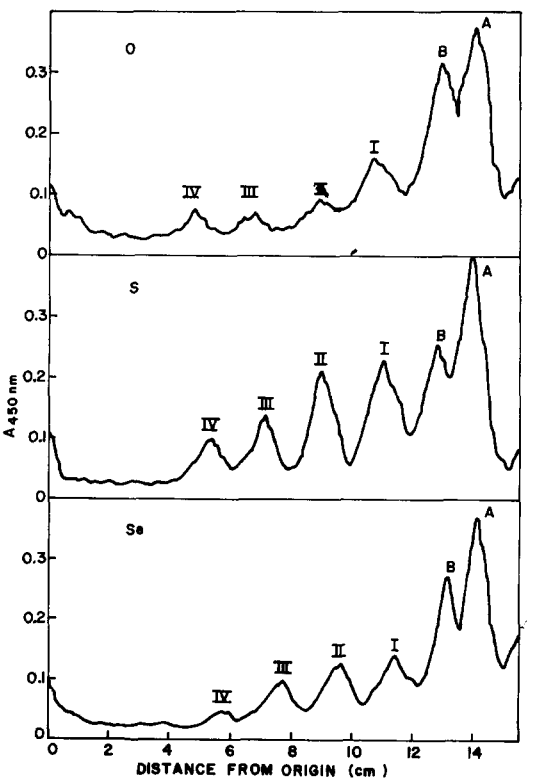
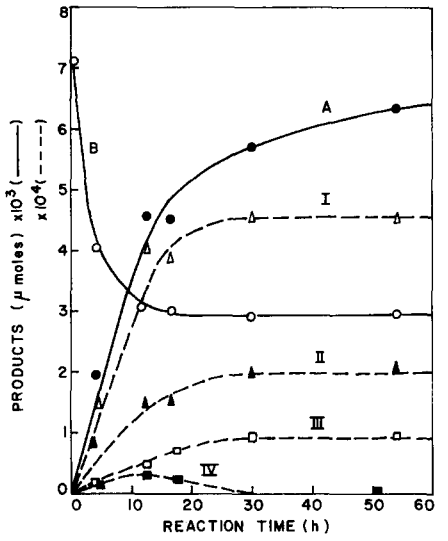


Fig. 4. Scanning records for thin-layer chromatographic analyses of lysozyme digests (17 h) of MO-NAG_2 (A), MS-NAG_2 (B) and MSe-NAG_2 (C).



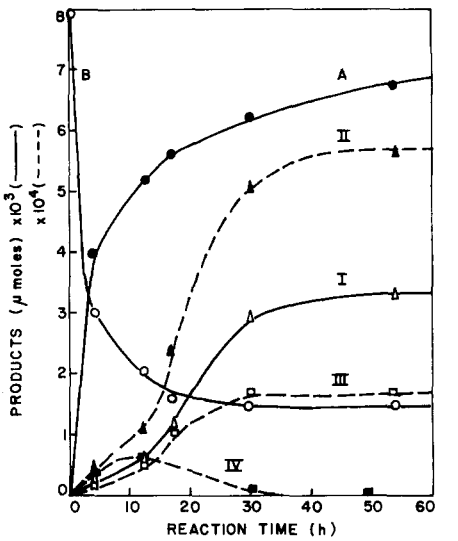


Fig. 5. Time course of lysozyme digestion of MO-NAG_2 (○—○) for MO-NAG (●—●), NAG (△—△), NAG_2 (▲- -▲), NAG_3 (□- -□), and NAG_4 (■- -■).

Fig. 6. Time course of lysozyme digestion of MS-NAG_2 (○—○) for MS-NAG (●—●), NAG (△—△), NAG_2 (▲- -▲), NAG_3 (□- -□) and NAG_4 (■- -■).

its isologs and Component B is the substrate. No aryl group-containing product with more than two NAG units was detected in the digests. Components I, II, III and IV are visualized by charting the plates with H_2SO_4 and are characterized as NAG, NAG_2, NAG_3 and NAG_4 with authentic compounds. NAG_3 and NAG_4 which were formed by the transglycosylation, were detected in the digests of all three substrates. *N*-Acetyl-

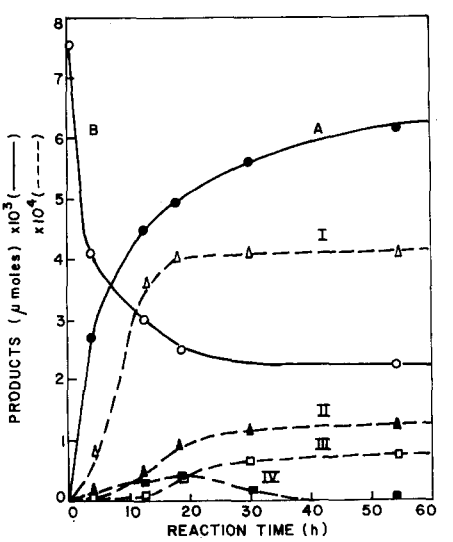


Fig. 7. Time course of lysozyme digestion of MSe-NAG_2 (○—○) for MSe-NAG (●—●), NAG (△- -△), NAG_2 (▲- -▲), NAG_3 (□- -□) and NAG_4 (■- -■).

chitooligoses with a higher degree of polymerization were not observed because the lysozyme digests were treated with chloroform and centrifuged prior to the analysis.

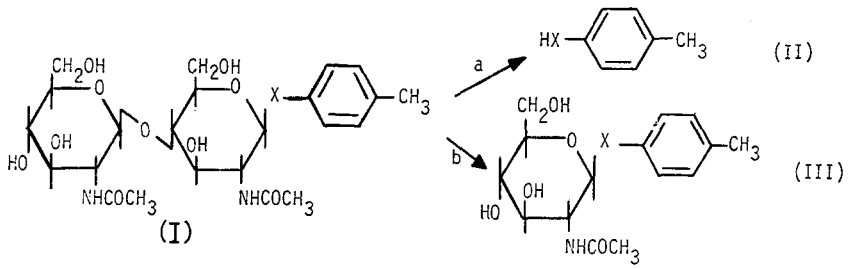
Time courses for lysozyme digestions of MO-NAG_2, MS-NAG_2 and MSe-NAG_2 are presented in Figs. 5–7. For MO-NAG_2 and MS-NAG_2 the major products, MO-NAG and MS-NAG were formed rapidly and accumulated as the reaction proceeded. In contrast, the formation of NAG, NAG_2 and NAG_3 appeared after a time lag. NAG_4 was formed at the early stage by the transglycosylation but was degraded by the subsequent hydrolysis. The reaction pattern of MSe-NAG_2 differs from those of MO-NAG_2 and MS-NAG_2 in that there was no detectable time lag for the formation of NAG, NAG_2 and NAG_3.

DISCUSSION

The active site of lysozyme is a cleft which can accommodate six pyranose rings at subsites designated A, B, C, D, E and F[6]. Synthetic substrates or *N*-acetyl chitooligoses with less than three pyranose rings bind preferentially in a nonproductive mode to subsites A-C of the enzyme[20–28]. The formation of nonproductive complexes can be studied by ultraviolet spectrophotometry[25,28] and NMR spectrometry[29,30]. The dissociation constants decrease with an increasing number of pyranose rings. Dissociation constants of aryl *N*-acetylchitobiosides are intermediate to those of NAG_2 and NAG_3 (ref. 29,30). suggesting contribution of the aryl ring, though to a lesser extent than the pyranose ring, to the binding of NAG_2. In contrast, the alkyl groups do not seem to promote the binding of glycosides[31].

In the acid-catalyzed hydrolysis of phenyl-β-D-glucopyranosides, the replacement of the glycosidic oxygen by sulfur or selenium has a rate-decreasing effect, whereas in the base-catalyzed hydrolysis, the glycosidic sulfur or selenium enhances the rate[32]. Similar isolog effects are observed in the acid and base-catalyzed hydrolyses of MO-NAG, MS-NAG and MSe-NAG. In the lysozyme catalysis, initial velocities for the liberation of phenols follow the order: $O \geqslant S > Se$. The substitution of the glycosidic oxygen with sulfur or selenium may affect the affinity of substrates with the enzyme (*i.e.* dissociation constants) and/or catalytic rates*.

If we consider reaction sequences by which the isologous substrates (I) yield phenols (II) and MX-NAG (III) as follows:



The relative rates for lysozyme-catalyzed hydrolyses of the glycosidic linkages bearing

* The analysis of lysozyme catalysis by the Michaelis–Menten equation has been reported[33,19]. This treatment which gives $S > O > Se$ for K_{cat} and $O > S > Se$ for K_m is, however, considered inadequate kinetically[34].

O, S and Se can be estimated by comparing the ratio $v(II)_X/v(III)_X$. $v(II)_X$ and $v(III)_X$ are initial velocities for the formation of (II) and (III) where X = O, S and Se*. The rate ratio for O:S:Se $\cong$ 20:5:3 which correlates with the relative order of the acid-catalyzed hydrolysis, is in agreement with the mechanism proposed by BLAKE *et al.*[6]. This involves the protonation of the glycosidic atom of substrates by Glu-35 to form a conjugated acid, followed by heterolysis to give a carbonium ion which is stabilized by Asp-52 carboxylate of the enzyme. The isolog effect in this study further substantiates the growing experimental evidence supporting the carbonium ion mechanism for lysozyme catalysis[35,36].

Synthetic substrates differ from natural substrates in that the former compounds do not possess reducing ends. It is possible that lysozyme-synthetic substrate complexes may exhibit more randomized patterns than those between lysozyme and natural substrates. This is reflected in the patterns of cleavage of NAG_3 and MO-NAG_2. NAG_3 is cleaved preferentially at the nonreducing glycosidic linkage[37,38] whereas the cleavage of MO-NAG_2 favors the aryl glycosidic linkage. In the transglycosylation of natural substrates, NAG_4 is one of the early products which accumulates[9], whereas NAG_4 acts as an intermediary product in the transglycosylation of synthetic substrates. Time courses of transglycosylation as shown in Figs. 5–7 for the isologs can best be explained by the difference in reactivities of the two glycosidic linkages and the subsequent transglycosylation. Phenols, MO-NAG, MS-NAG and MSe-NAG are presumably formed by the hydrolysis. They are accumulated since they are poor glycosyl acceptors. The accumulation of NAG, NAG_2 and NAG_3 from MO-NAG_2 and MS-NAG_2 is preceded by a time lag suggesting that these products are formed by way of the transglycosylation of which NAG_4 is the intermediary product.

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* The acceptor activities of (II) are negligible compared with glycoses[35]. Indeed, no aryl group-bearing product with more than two NAG units was detected in the digests. Furthermore, the acceptor activties of (III) where X = O, S or Se are assumed to be the same because X is distal to the acceptor site at 4′-OH.

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