Substrate Hydroxyl Groups Are Involved in the Ionization of Catalytic Carboxyl Groups of Aspergillus niger α-Glucosidase

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Abstract: Kinetic studies on hydrolysis of the substrates p-nitrophenyl α-D-glucopyranoside (Glcα-O-p-NP), methyl β-D-maltoside (Malβ-O-Me), and their 2- or 3-deoxy analogs, were conducted to investigate the interaction between sugar hydroxyl groups and catalytic carboxyl groups of Aspergillus niger α-glucosidase (ANGase). Optimal pH value for the reaction between the enzyme and Glcα-O-p-NP (pH 4.4) was different than that for both its 2- and 3-deoxy analogs (pH 5.6), suggesting that ionization of two catalytic carboxyl groups of ANGase is affected by glycon OH-2 and -3 groups on Glcα-O-p-NP. Through hydrolysis of the glucosides we elucidated pKc and pKs, for each carboxyl group of the enzyme using Dixon-Webb semi-log plots. The pKc and pKs values were different for Glcα-O-p-NP, but identical for the 2- and 3-deoxy analogs. Similar results were obtained in the reaction involving Malβ-O-Me and its 2-deoxy analog as substrates, indicating that the glycon OH-2 and -3 groups of the glucoside are intimately involved in the ionization of two catalytic carboxyl groups of ANGase, while aglycon hydroxyl groups do not appear essential for the ionization of the carboxyl groups.

Key words: Aspergillus niger α-glucosidase, carbohydrate-enzyme interaction, Dixon-Webb semi-log plots, ionization constants, monodeoxy glucoside

Carbohydrate hydrolases are widely distributed in microorganisms, plants, insects, and mammals. These enzymes are classified on the basis of their modes of action (exo- or endo-type), sugar specificity, form of sugar chain, and configuration (α- or β-anomer) and position of the glycosidic linkage. Hydrogen bonding interactions between the amino acids involved in the active site of these enzymes and the hydroxyl groups of their sugar substrates are important in the formation of the enzyme-substrate (ES) complex. In fact, with some enzymes, systematic hydrogen bondings between their active sites and their substrates have been estimated from three-dimen-sional structure analyses. For example, extensive hydrogen bond formation was indicated between the active site of glucoamylase (EC 3.2.1.3) from the mold Aspergillus niger and methyl α-maltoside or -isomaltoside.¹,² Studies using mono-deoxyninated substrates demonstrated that specific hydroxyl groups of the glycoside play an essential role in ES complex formation with glucoamylases,¹⁻⁴ α-glucosidases (EC 3.2.1.20),⁴⁻⁷ β-glucosidases (EC 3.2.1.21),⁴⁻⁷ α-galactosidases (EC 3.2.1.22),⁴⁻⁷ β-galactosidases (EC 3.2.1.23),⁴⁻⁷ and α-mannosidases (EC 3.2.1.24)⁷ from various organisms. Active site amino acids involved in substrate binding have been identified by studies of site-directed mutagenesis.⁸,⁹ Thus, investigations into interactions between an enzyme and its sugar substrate focus on hydrogen bond formation. However, Notenboom et al. reported that the nonreducing-end sugar (glycon) OH-2 group of aryl β-cellobioside promotes transition-state stabilization of reaction intermediates during hydrolytic reactions of the bacteria Cellulomonas fimii β-glucosidase.²⁰ Moreover, Frandesen et al. also indicated that OH-2 and -3 groups of the reducing-end ring (aglycon) of the substrate methyl a-isomaltoside contribute to transition-state stabilization in yeast α-glucosidase, while all glycon hydroxyl groups of the substrate are intimately involved in the formation of hydrogen bonds with the enzyme.⁵ Using substrates partially modified at their glycon portions, we have studied substrate specificities of various types of α-glucosidases.⁶,⁷ Thus, through these studies, we predicted that some properties of the hydrolytic actions of exo-glucosidases are controlled by the glycon hydroxyl groups of their substrates. On the basis of this hypothesis, we investigated the role of the glycon hydroxyl groups of the glycoside during α-glucosidase action by kinetic studies on the hydrolysis of partially deoxyninated glucosides.

We chose A. niger α-glucosidase (ANGase) for this study because of its strong hydrolytic activity against 2- and 3-deoxy glucosides modified at the glycon portion, al-
though it possesses no activity toward 4- and 6-deoxy glucosides. This enzyme belongs to the glycoside hydro- lase family 31 on the basis of its amino acid se-
quence.21–23 Catalytic ionizable groups on the active site of ANGase were identified as a pair of carboxyl groups derived from two aspartic acid residues.24–26 Hydrolysis of a-glucoside by the enzyme occurred with retention of stereochemistry at the anomeric center.27 In general, the reaction mechanism of the retaining a-glucosidases with a pair of catalytic carboxyl groups in their active sites is a double displacement process.26,28,29 Forms of these carboxyl groups in the active enzyme are -COO\(^{-}\) (nucleophile) and -COOH (proton donor). Indication exists that family 31 a-glucosidases from sugar beet and yeast Schizosaccharomyces pombe also hydrolyze the a-glucosidic substrates using a pair of catalytic carboxyl groups in their single active site.30,31 Previously, we investigated \(K_m\) and \(k_{cat}\) values of ANGase for the hydrolysis of \(p\)-nitrophenyl \(a\)-d-glucopyranoside (Glc\(a\)-\(O\)-pNP) and its 2- and 3-deoxy analogs (2D-Glc\(a\)-O-pNP and 3D-Glc\(a\)-O-pNP, respectively).32 It is clear that hydrolysis of these substrates was catalyzed by the same catalytic groups of the enzyme. In this study, we investigate the effect of glycon hydroxyl groups of glucoside toward the catalytic carboxyl groups of ANGase by kinetic studies of the enzymatic hydrolysis of two types of substrates, synthetic ayl glucosides Glc\(a\)-O-pNP and its 2- and 3-deoxy analogs, and the disaccharide derivatives methyl \(\beta\)-maltoside (Mal \(\beta\)-O-Me) and its 2-deoxy analog modified at the glycon portion (2D-Mal\(\beta\)-O-Me) (Fig. 1).

![Fig. 1. The structures of substrates used in this study.](image)

**MATERIALS AND METHODS**

**Enzyme and substrates.** Transglucosidase L, a crude preparation of ANGase, was a gift from Amano Enzyme (Nagoya, Japan). ANGase was purified from this crude preparation as previously reported,33 and the purified enzyme confirmed to be homogeneous using SDS- and native-polyacrylamide gel electrophoresis. Solution concentration of the enzyme was measured spectrophotomet-
ically using an adsorption coefficient \(\varepsilon\) of 17.7 cm\(^{-1}\) for 1% (w/v) protein at 280 nm. Glc\(a\)-O-pNP was pur-
chased from Tokyo Kasei Kogyo (Tokyo, Japan), and was purified by recrystallization from EtOH. 2D-Glc\(a\)-O-pPNP34 and 3D-Glc\(a\)-O-pPNP35 were synthesized according to procedures described in our previous papers. Mal\(\beta\)-O-Me was prepared by the method reported.36 Synthesis of 2D-Mal\(\beta\)-O-Me was conducted as described in this study.

**Synthesis of 2D-Mal\(\beta\)-O-Me.** Synthesized compounds were characterized by \(^1\)H NMR and MS. Melting points were determined with a Yamato Model MP-21 capillary apparatus and are uncorrected. Optical rotations were measured with a JASCO P-1020 polarimeter at 25°C. \(^1\)H-NMR spectra were recorded with a Varian VXR-400 spectrometer. Chemical shifts are expressed in ppm down-
field shift from MeSi. Mass spectra were obtained with a JEOL JMS-SX102A instrument under positive FAB conditions.

To a mixture of 2-deoxy-1,3,4,6-tetra-O-acetyl-\(\alpha\)-arabi-
no-hexose (2.0 g, 6.02 mmol), prepared by acetylation of 2-deoxy glucose (Sigma-Aldrich, St. Louis, USA) in a mixture of Ac\(2\)O and pyridine, and methyl 2,3,6-tri-O-
benzyl-\(\beta\)-d-glucopyranoside37 (3.08 g, 6.63 mmol) in dry toluene (100 mL) was added trimethylsilyl trifluoromethanesulfonate (1.16 mL, 6.02 mmol) at room temperature. The mixture was stirred for 30 min and poured into saturated NaHCO\(_3\) solution, washed with brine, and then concen-
trated after drying over Na\(_2\)SO\(_4\). The product was purified by column chromatography on silica gel (Silica Gel 60, 230–400 mesh, E. Merck, Darmstadt, Germany) using hexane/EtOAc (2 : 1, v/v) as a mobile phase to afford 2.43 g (3.30 mmol) of methyl 3,4,6-tri-O-acetyl-2-deoxy-
\(\alpha\)-d-arabinopyranosyl-(1-4)-2,3,6-tri-O-benzyl-\(\beta\)-d-
glucopyranoside as a syrup: \([\alpha]\)\(_D\) + 40.2° (c 0.492, CHCl\(_3\)); FABMS: 737 [M\(^+\)]\(^-\); \(^1\)H NMR (CDCl\(_3\)): (gly-
con) \(\delta\) 1.61 (ddd, 1H, \(J_{2ax,5} = 2.8\) Hz, \(J_{2ax,eq,6} = 12.0\) Hz, \(J_{2ax,3ax} = 5.2\) Hz, H-2ax), 1.98 (s, 3H, OCOCH\(_3\)), 2.02 (s, 6 H, \(2\times\) OCOCH\(_3\)), 2.02 (ddd, 1H, \(J_{1ax,4,5} = 1.2\) Hz, \(J_{5,6} = 2.8\) Hz, H-2eq), 3.80 (dd, 1H, \(J_{6ax,2} = 2.0\) Hz, \(J_{6ab,2} = 12.0\) Hz, H-6a), 3.88 (ddd, 1H, \(J_{6a,9,6b} = 9.6\) Hz, \(J_{6ab,9,6b} = 4.0\) Hz, H-5)), 4.15 (dd, 1H, H-6b), 4.89 (t, 1H, \(J_{1,9} = 9.6\) Hz, H-4), 5.19 (d, 1H, H-3), 5.42 (dd, 1H, H-1), (aglycon) \(\delta\) 3.42 (dd, 1H, \(J_{2,3} = 8.0\) Hz, \(J_{2,5} = 8.8\) Hz, H-2), 3.50 (ddd, 1H, \(J_{5,6a} = 9.2\) Hz, \(J_{5,6b} = 6.0\) Hz, \(J_{6a,6b} = 2.0\) Hz, H-5), 3.59 (s, 3H, OCH\(_3\)), 3.60 (t, 1H, \(J_{1,9} = 8.8\) Hz, H-3), 3.66 (dd, 1H, H-4), 3.67 (ddd, 1H, \(J_{6ab,11,6a} = 11.2\) Hz, H-6a), 3.77 (dd, 1H, H-6b), 4.33 (d, 1H, H-1), 4.57 (d, 1H, \(J = 12.4\) Hz, CH\(_2\)Ph), 4.59 (d, 1H, \(J = 11.2\) Hz, CH\(_2\)Ph), 4.65 (d, 1H, \(J = 10.8\) Hz, CH\(_2\)Ph), 4.66 (dd, 1H, \(J = 12.0\) Hz, CH\(_2\)Ph), 4.91 (d, 1H, \(J = 11.2\) Hz, CH\(_2\)Ph), 4.99 (d, 1H, \(J = 11.6\) Hz, CH\(_2\)Ph), 7.19–7.38 (m, 15H, aromatic H).

Next, the isolated product (2.0 g, 2.71 mmol) was dis-
solved in a mixture of NEt\(_3\)/H\(_2\)O/MeOH (1 : 2 : 7, v/v/v). After stirring at room temperature for 2 days, the sol-
vent was evaporated to dryness and the product recrystallized from hexane/EtOAc to afford 1.38 g (2.26 mmol) of methyl 2-deoxy-\(\alpha\)-d-arabino-hexopyranosyl-(1-4)-2,3,6-
tri-O-benzyl-\(\beta\)-d-glucopyranoside as a crystalline solid. To a solution of this compound (1.10 g, 1.80 mmol) in MeOH (80 mL) was added 0.3 g of palladium hydroxide (Pd-OH, 20% on carbon) and 0.1 mL of 1 N HCl. After the mixture was stirred at room temperature for 20 h un-
der \(\text{H}_2\), the Pd-OH was removed by filtration and the sol-
vent evaporated to dryness. The residue was dissolved in H\(_2\)O (30 mL) and the solution washed with EtOAc to re-
move benzyl alcohol. The aqueous layer was concentrated and the product lyophilized to afford 0.512 g (1.50 mmol) of methyl 2-deoxy-\(\alpha\)-d-arabino-hexopyranosyl-(1-4)-\(\beta\)-d-
glucopyranoside (2D-Mal\(\beta\)-O-Me): mp 165.5–170°C; \([\alpha]\)\(_D\) + 68.8° (c 0.532, H\(_2\)O); FABMS: 341 [M\(^+\)]\(^-\); \(^1\)H NMR (D\(_2\)O): (glycon) \(\delta\) 1.74 (ddd, 1H, \(J_{2,3ax} = 3.6\) Hz, 10.1, No. 1 (2004)
**Carbohydrate-Active Center Interaction in A. niger α-Glucosidase**

Kinetic studies of ANGase during hydrolysis of Glcα-O-pNP and its 2- and 3-deoxy analogs were conducted in the pH range of 2.5–7.5 in a 1:2 dilution of McIlvaine buffer (1/2 McIlvaine buffer). After addition of the enzyme to the reaction mixture in a final volume of 0.5 mL, the mixture was incubated at 37°C for 10 min, then measured spectrophotometrically at 505 nm. It was difficult to determine 2-deoxy glucose by this method, because of the lower activity of the glucose oxidase in the kit against the monodeoxy sugar. Therefore, for determining the amount of 2-deoxy glucose, an additional 11 units of A. niger glucose oxidase (Sigma-Aldrich, St. Louis, USA) was added to the assay mixture, followed by incubation at 37°C for 50 min. Values of V and K<sub>m</sub> at various pHs were obtained from double reciprocal plots of the reaction curves. Values of pK<sub>e</sub> and pK<sub>e2</sub> for the two catalytic carboxyl groups of ANGase were obtained from Dixon-Webb semi-log plots, based upon these V and K<sub>m</sub> values.

**RESULTS AND DISCUSSION**

**pH profile of ANGase hydrolytic activity.**

We measured the pH dependence of the hydrolytic activity of ANGase against Glcα-O-pNP and its 2- and 3-deoxy analogs. Previously, we had elucidated the Km values of the enzyme for these three substrates at pH 4.0, and confirmed values of 0.59, 6.09 and 10.2 mM for Glcα-O-pNP, 2D-Glcα-O-pNP and 3D-Glcα-O-pNP, respectively. Taking into consideration these Km values and solubility of the deoxy substrates, pH dependence of the enzyme activity against these substrates was investigated at concentrations of 1/2 Km. Optimal pH for the ANGase reaction with Glcα-O-pNP was 4.4, while the pH for both deoxy analogs was 5.6 (Fig. 2). Thus, the optimal pHs for this enzyme were different for Glcα-O-pNP and for its 2- and 3-deoxy analogs. The activity of ANGase is influenced by the dissociation states of its catalytic carboxyl groups, and their states differ with pH. The pH profiles shown in Fig. 2 indicate that the degree of dissociation of the carboxyl groups is different in Glcα-O-pNP binding than in its deoxy analog binding, due to the glycon OH-2 and -3 groups of Glcα-O-pNP.

To gain further insight into the effect of the sugar hy-
droxyl groups toward the catalytic carboxyl groups of ANGase, kinetic studies of the hydrolytic action of the enzyme were undertaken using Glcα-O-pNP, Malβ-O-Me, and their 2- or 3-deoxy analog.

**Kinetic study of the hydrolytic reaction with ANGase.**

Generally, in an enzyme having a pair of carboxyl groups as catalytic ionizable groups (nucleophile and proton donor), three ionized forms follow a two-step mechanism in the dissociation process (Fig. 3).\(^\text{34,36}\) On the basis of this mechanism, we investigated pKe (in the free enzyme) and pKes (in the ES complex) of the two catalytic carboxyl groups of ANGase during hydrolysis of Glcα-O-pNP and its 2- and 3-deoxy analogs, using Dixon-Webb semi-log plots. Figure 4 shows the plots of pKm, log V, and log V/Km versus pH for Glcα-O-pNP (Fig. 4A) and for 2D-Glcα-O-pNP (Fig. 4B). Pattern of the plot for 3D-Glcα-O-pNP was the same as that for 2D-Glcα-O-pNP. With Glcα-O-pNP, pKm decreases not only on the acidic side but also on the alkaline side, whereas those for 2- and 3-deoxy analogs were constant over various pHs. The plots indicate that each of the two catalytic carboxyl groups of ANGase possesses different values of Ke and Kes with Glcα-O-pNP and corresponding values for each deoxy analog. Table 1 shows pKe and pKes values of the two catalytic groups for the three substrates. With Glcα-O-pNP as a substrate, pKe1 and pKes1 (for the nucleophile) were 3.5 and <2.6; and pKe2 and pKes2 (for the proton donor) were 6.4 and 6.8. The values of pKe and pKes for the two deoxy analogs were: (2D-Glcα-O-pNP) pKe1 and pKes1, 3.4; pKe2 and pKes2, 6.4; (3D-Glcα-O-pNP) pKe1 and pKes1, 3.5; pKe2 and pKes2, 6.4. Values of pKes-pKe1 and pKes-pKe2 with Glcα-O-pNP were <−0.9 and 0.4, respectively. With 2D- and 3D-Glcα-O-pNP, no difference was found between pKe1 and pKes1 (or pKe2 and pKes2) for the carboxyl groups, indicating that OH-2 and -3 of Glcα-O-pNP are deeply involved in the ionization of the catalytic carboxyl groups of ANGase. For practical purposes, both hydroxyl groups significantly promote the dissociation of the nucleophile, while ionization of the proton donor is suppressed by them. We believe that the greater activity of ANGase for Glcα-O-pNP at lower pH values is due to enhanced dissociation of the acidic side catalytic carboxyl group.

To investigate the effect of aglycon hydroxyl groups for the catalytic carboxyl groups of ANGase, kinetic studies were conducted using Malβ-O-Me and its 2-deoxy analog modified at the glycon. Kinetic parameters (Km and V) of the enzyme for these substrates at pH 4.1 were: Malβ-O-Me, 1.25 mM and 147 μmol/min/mg protein; 2D-

![Fig. 3. Three ionization forms of the two catalytic carboxyl groups of glycoside hydrolase in a two-step mechanism.](#)

![Fig. 4. Plots of pKm, log V and log V/Km versus pH for hydrolysis of Glcα-O-pNP and 2D-Glcα-O-pNP by ANGase.](#)
Table 1. $pK_e$ and $pK_{es}$ values of catalytic carboxyl groups of ANGase during hydrolysis of Glcα-O-βNP and its 2- and 3-deoxy analogs, and difference between $pK_e$ and $pK_{es}$ values.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nucleophile</th>
<th>Proton donor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pK_{es}$</td>
<td>$pK_{es}−pK_e$</td>
</tr>
<tr>
<td>Glcα-O-βNP</td>
<td>3.5</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>2D-Glcα-O-βNP</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>3D-Glcα-O-βNP</td>
<td>3.5</td>
<td>3.5</td>
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</tbody>
</table>

Concentrations of 3D-Glcα-O-βNP in the reaction mixtures were 0.2–5.0 mM. Amounts of the enzyme protein added to the reaction mixtures were 0.72–3.0 μg for 3D-Glcα-O-βNP. Other reaction conditions for 3D-Glcα-O-βNP and 2D-Glcα-O-βNP (in Fig. 4).

Table 2. $pK_e$ and $pK_{es}$ values of catalytic carboxyl groups of ANGase during hydrolysis of Malβ-O-Me and its 2-deoxy analog, and difference between $pK_e$ and $pK_{es}$ values.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nucleophile</th>
<th>Proton donor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pK_{es}$</td>
<td>$pK_{es}−pK_e$</td>
</tr>
<tr>
<td>Malβ-O-Me</td>
<td>3.2</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>2D-Malβ-O-Me</td>
<td>3.3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Kinetic study of the enzymatic reaction was conducted in the pH range of 2.6–7.0 at 37°C in 0.15 mM of 1/2 McIlvaine buffer. $V$ and $K_m$ were obtained from double reciprocal plots of the reaction curves. Least squares fitting was applied to the data points. Substrate concentrations in the reaction mixtures were 0.3–4.0 and 0.5–5.0 mM for Malβ-O-Me and 2D-Malβ-O-Me, respectively. Amounts of the enzyme protein added to the reaction mixtures were 0.03–3.3 μg for Malβ-O-Me and 2.0–4.5 μg for 2D-Malβ-O-Me, respectively. To obtain $pK_e$ and $pK_{es}$ for the two catalytic carboxyl groups of ANGase for these substrates, Dixon-Webb semi-log plotting were done using $V$ and $K_m$ values derived experimentally.

Malβ-O-Me, 5.33 mM and 41.7 μmol/min/mg protein. Table 2 shows $pK_e$ and $pK_{es}$ values of these two ANGase catalytic groups for the two substrates. The $pK_e$ and $pK_{es}$ values for Malβ-O-Me were: $pK_e$ and $pK_{es}$, 3.2 and <2.6; $pK_{es}$ and $pK_{es}$, 6.0 and 6.3. When the 2-deoxy analog was used as a substrate, the following values were obtained: $pK_e$ and $pK_{es}$, 3.3; $pK_{es}$ and $pK_{es}$, 6.1. Values of $pK_{es}−pK_e$ and $pK_{es}−pK_{es}$ for Malβ-O-Me were <−0.6 and 0.3, respectively. In contrast, there was no difference between $pK_e$ and $pK_{es}$ (or $pK_{es}$ and $pK_{es}$) of the carboxyl groups for 2D-Malβ-O-Me. These data indicated that the glycon OH-2 groups of Malβ-O-Me are involved in the dissociation of the two catalytic carboxyl groups of ANGase, similar to the data found for Glcα-O-βNP, while its aglycon hydroxyl groups do not participate in the ionization of these carboxyl groups.

With the strong effect of the glycon OH-2 and -3 groups of the glycoside toward the nucleophilic carboxyl group of Asp 224β of ANGase, the mechanism of the interaction is unclear. However, it is obvious that both hydroxyl groups are involved in promoting dissociation of this carboxyl group, and this promotion results in the greater activity of the enzyme for glucoside at lower pH values. To elucidate the interaction between sugar hydroxyl group and active center carboxyl groups of ANGase, further studies using the technique of site-directed mutagenesis and three-dimensional structure analysis of the proteins should be done with ANGase. Now we have plans to make some mutant enzymes and react them against the deoxygenated glucosides.

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基質水酸基が Aspergillus niger α-グルコンダーゼの活性中心カルボキシル基の解離に与える影響

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糖基質水酸基が Aspergillus niger α-グルコンダーゼ（ANGase）の二つの活性中心カルボキシル基に与える影響を調べるために、ANGaseによるp-ニトロフェニル-α-D-グルコンピラノン（Glc-pNP）、メチルβ-D-マルトシド（Malβ-O-Me）とそれらの2-および3-デオキシ糖に対する加水分解反応の速度論的解析を行った。まず初めに、ANGaseによるGlc-pNPおよびその2、または3-デオキシ糖の加水分解反応の至適pHを求めたところ、Glc-α-pNPに対してはpH 4.4であったが、2-および3-デオキシ糖についてはpH 5.6と、両者にかなりの違いがみられ（Fig. 2）。このことから、Glc-α-pNPの2位および3位水酸基はANGaseの二つの活性中心カルボキシル基の解離に影響を与えていることが示唆された。また、本酵素のこれらの基質に対する反応について、酵素活性中心のα-のカルボキシル基のpKa値およびpKε値をディクソン-ウェブ セミログループ分析し求められた（Fig. 4）。その結果Glc-α-pNPに対しては2-および3-デオキシ糖に対してはpKa値が一致していた（Table 1）。さらに同様の結果がMalβ-O-Meとその2-デオキシ糖についても観測された（Table 2）。以上のことから、基質グリコン2位および3位水酸基はANGaseの二つの活性中心カルボキシル基の解離に大きな影響を与えていることが示唆された。一方、グリコン部分の糖酸基は、本酵素の活性中心の解離には必須ではないことが示唆された。