Glycolipid Enzyme Models. XIV
Effects of pH on Catalytic Activity

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Abstract: Z-Phenylalanine and Z-methionine p-nitrophenyl esters were hydrolyzed using a glycolipid hydrolase model at various pH. The glycolipid showed pH-dependence of hydrolyzing ability, as also noted for α-chymotrypsin, the profile being a mountain shaped curve peaking at pH 8, regardless of the presence or absence of a reaction field of phosphatidylcholine. The substrate-recognition ability of the glycolipid exhibits the reverse tendency to pH-dependence of the hydrolyzing ability. These findings are explained by increase in active species and decrease in vesicle membrane stability with pH.

Key words: recognition, hydrolase model, vesicle glycolipid, pH, substrate

1 Introduction

Recently, studies on the relationship between function and structure of an enzyme, which catalyses chemical reactions in a living creature, and on the development of excellent catalysts having the function resembling an enzyme, have been reported by many investigators. Almost all of these reports treat the intended phenomena and reactions with kinetic analyses. The kinetics of enzymatic reactions were originated in the discovery of Henri in 1902. He found that the hydrolysis of sucrose by an invertase of yeast proceeds in a different kinetic from that of acidic hydrolysis reported by Wilhelmy in 1850. The research which we often cite as a historical bible for enzymatic kinetics, however, is a hydrolysis of sucrose by invertase reported by Michaelis and Menten in 1913. This is due to the fact that the concept of pH had not been present in the age of Henri, who did not take into consideration the important effect of pH on the rate of an enzymatic reaction. An enzyme is a highly functionalized catalyst, but is very sensitive to pH change. That is, an enzyme is denatured by an extreme change of pH to lose its biological activity. For example, α-chymotrypsin can digest proteins in an intestine and work most effectively around pH 7, which corresponds to the pH in the cells, and decreases its activity above and below the optimum pH1). Another similar hydrolase digesting proteins of food in stomach can work best at the lower pH values as seen in the stomach. Generally speaking, many enzymes, especially hydrolases, have been investigated with respect to the relationship between the activity and pH, and the pH-dependence of enzymatic functions was discussed in relationship with the conformational change of enzyme itself, with pH value of a surrounding medium. An enzyme, however, does not exist independently, but exists on or in a biomembrane as shown in a mosaic model proposed by Singar-Nicolson2). The effect of pH value on enzyme activities, therefore, should be discussed in combination of an enzyme with the surrounding circumstance.

In a series of our papers3),4), it has been reported that a kind of synthetic glycolipid is a hydrolase model with a substrate-recognizing ability similar to that of α-chymotrypsin. A quasi-biomembrane consisting of the glycolipid and a synthetic phospholipid (reaction field) was prepared and utilized to examine the effect of the reaction field on activity of an enzyme model. In addition, the effect of membrane fluidity6) was also studied to elucidate the enzyme activity.

In this study, the response of a combination of enzyme model and reaction field to an external stimulation, such as pH value, is investigated using
a biomembrane model, in order to estimate the behavior of an enzyme responding to environmental change in a living creature.

2 Experiments

2-1 Reagents

2-1-1 Substrates and catalyst

Substrates used in this study were p-nitrophenyl esters of Z-L-amino acids (Z means carbobenzoxy group) with side chains of different hydrophobic nature\(^5\) (Fig. 1). An ester, for example, of phenylalanine is designated Z-L-Phe-ONp. They were synthesized by methods described in a previous paper\(^6\). A catalyst was the synthetic glycolipid (Man(Lau)\(_2\)), which has mannose and di-n-dodecylamine residues as hydrophilic and hydrophobic moieties, respectively, and can form vesicles in water by itself.

2-1-2 Synthesized phospholipids

A biomembrane model was prepared using synthetic phospholipids as shown in Fig. 2. The phospholipid has two C\(_{18}\) saturated hydrocarbon chains derived from stearic acid (satd. PC) or two C\(_{18}\) unsaturated hydrocarbon chains from linseed oil (unsatd. PC) as hydrophobic moiety. The synthesis was conducted by a method described in a previous paper\(^7\).

2-2 Procedures

2-2-1 Hydrolyses

In order to examine a pH-depending behavior of a model enzyme in biomembrane, a catalytic vesicle solution of glycolipid with phospholipid was prepared by a thin film method. A 0.01 M solution (50 \(\mu\)L) of the glycolipid in methanol and 0.01 M solution (5 \(\mu\)L) of a synthetic phospholipid in acetone were charged into a test tube. After removal of solvent in vacuo, a buffer solution (30 mL) was added, and was sonicated for 15 min above the phase transition temperature of a phospholipid to prepare a vesicle solution (when a saturated or unsaturated synthetic phospholipid was used, the preparation was carried out at 90 or 25 °C, respectively), which was used as a catalyst. When a vesicle of glycolipid without phospholipid was formed, on the other hand, 0.01 M solution (50 \(\mu\)L) of the glycolipid in methanol was poured into a buffer solution (30 mL) and then sonicated at 25 °C for 15 min to prepare a vesicle solution as a catalyst.

The solution (3 mL) was put in a quartz cell having width of 1 cm, and 0.001 M solution (12.5 \(\mu\)L) of an amino acid ester in acetone was injected as substrate. The hydrolysis was pursued by a colorimetric measurement of the amount of liberated p-nitrophenolate ion at 400 nm using a JASCO V-520 spectrophotometer. The measurements were carried out repeatedly in solutions buffered with potassium hydrogen phthalate-NaOH (for pH 4.0 ~ 6.0) or tris(2-amino-2-hydroxymethyl-1,3-propanediol)-HCl (Tris) (for

* The term “fluidity” in this paper means sway or movement of components constituting a membrane and is used in contrary sense to “gel”, in which components are rather fixed.
3 Results and Discussion

3-1 Effect of Buffers

Spontaneous hydrolysis rate of an amino acid ester was measured in a reaction solution without glycolipid at various pH values, before the pH-dependence of catalytic activity of the glycolipid was determined. The spontaneous hydrolyses of Z-L-Phe-ONp are shown in Fig. 3. The hydrolysis rate was increased suddenly at a pH value of about 9.0, due to alkaline hydrolysis. It is considered, therefore, that a pH over 9.0 is inadequate for the study on hydrolyses catalyzed by a glycolipid. Thus, the measurements in this study were carried out over the pH range of 4.0 to 9.0. In order to set pH at 4.0~9.0, two kinds of buffer solutions were used (potassium hydrogen phthalate-NaOH for

<table>
<thead>
<tr>
<th>Buffer Solution</th>
<th>$k_2$ (M$^{-1}$sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium hydrogen phthalate-NaOH (pH 5.86)</td>
<td>442</td>
</tr>
<tr>
<td>Sodium cacodylate-NaOH (pH 5.82)</td>
<td>420</td>
</tr>
<tr>
<td>Tris-HCl (pH 7.00)</td>
<td>627</td>
</tr>
<tr>
<td>Sodium cacodylate-NaOH (pH 7.03)</td>
<td>639</td>
</tr>
</tbody>
</table>

**Table 1** Comparison of hydrolyzing activities in different buffer solutions.

Fig. 3 Spontaneous Hydrolyses of Z-L-Phe-ONp (in buffer solution, at 25°C).
pH 4.0–6.0 and tris(2-amino-2-hydroxymethyl-1,3-propanediol)-HCl for pH 7.0–9.0). In these cases, the effect of a kind of buffer agents on the catalytic activity of a glycolipid was checked by sodium cacodylate-NaOH having a buffer capacity which covers pH ranges of the both buffer solutions (pH 5.8–7.0). Shown in Table 1 are second-order rate constants ($k_2$) in hydrolyses of Z-L-Phe-ONp. Almost the same $k_2$ values were obtained using different buffers at pH's of ca 5.8 and 7.0. Phthalate and Tris buffer solutions, therefore, are not considered to affect the hydrolyses of amino acid esters catalyzed by a glycolipid.

3.2 Relationship between pH and Catalytic Activity

Phospholipids are essential components of a biomembrane. Phosphatidylcholine (diacyl-glycerol-phosphoryl-choline) exists in the largest amount among phospholipids. Vesicles of glycolipid and synthetic phospholipid in mole ratio of 10 : 1 were prepared and used as biomembrane model in this study. Shown in Fig. 4 is the pH-dependence of catalytic activities of a glycolipid in hydrolyses of Z-L-Phe-ONp. Unsaturated phospholipid enhanced the activity of glycolipid, while saturated one lowered the activity. Considering the remarkable difference based on the kind of phospholipids, the reaction fields, which they form, are estimated to affect functions of a glycolipid considerably. In a previous paper, it was reported that the activity of glycolipid is changed dramatically below and above the phase transition temperature of coexisting phospholipid, even if it exists in the amount of only 10%, and that this is ascribable to gel and liquid-crystalline (fluidized) states of reaction fields.

The phase transition temperatures (Tc) of the saturated and unsaturated ether-type synthetic phosphatidylcholines used in this study were 60.0 and −25.0 °C, respectively. The vesicle membrane containing synthetic saturated phospholipid, thus, is estimated to be in a gel state at the temperature of 25 °C, at which measurements of hydrolyses were conducted. On the other hand, a bilayer membrane consisting of glycolipid and synthetic unsaturated phospholipid would be in a liquid-crystalline state. The activity of glycolipid is considered to be increased in fluidized reaction field.

It is also found that the summits of pH profiles of the catalytic activity were at about pH 8, regardless of the state or kind of vesicle membranes (Fig. 4). But the change of $k_2$ with pH was smaller in case of mixed vesicles of phospholipid.
and glycolipid. That is, a phospholipid seems to relax a stimulation from the surroundings.

As stated above, the pH-profile of catalytic activity of a glycolipid seemed independent of coexistence of a phospholipid. Why, then, is the activity of a glycolipid affected by pH values? To clarify this point, the pH-dependence of catalytic activity was examined kinetically in more detail. The pH-dependences of \( k_2 \) and \( K_m \), in the case of Z-L-Phe-ONp, are shown in Fig. 5. The pH-dependence of \( 1/K_m \) was changed in parallel with that of \( k_2 \). In general, Michaelis constant (\( K_m \)) is used as an index representing the affinity of an enzyme with a substrate. Thus, the result shown in Fig. 5, which agrees well with that reported in a previous study\(^5\), shows that the hydrolyzing process of amino acid esters is controlled by the uptake step of a substrate. Taking this fact into consideration, the pH value is estimated reasonably to change the state of a vesicle membrane, followed by the change of an affinity of the membrane with a substrate, namely, of an uptake rate of a substrate.

In general, it is understood that an pH-dependence of enzymes is based on a complicated combination of a few factors. For example, the pH value affects dissociation states of amino acid residues, which may take part in the reaction as an active center, and/or maintains the conformational stability of an enzyme to provide an optimum activity. In this study, some of hydroxyl groups of a sugar moiety on vesicle surface are known to be dissociated to form active sites\(^9\). The surface state, thus, is naturally affected by the pH value of surroundings. This must be one of important factors determining the pH-dependence of the activity of a glycolipid.

In order to study, in more detail, the effect of pH on the uptake of a substrate, substrate selectivity was examined by changing pH values. p-Nitrophenyl esters of L-methionine and L-phenylalanine were selected as substrate. These have side chains of different degrees of hydrophobicity; the former ester is more hydrophilic, compared with the latter one. Shown in Fig. 6 are \( k_2 \) values and substrate-selective ratios (\( k_2,\text{phe}/k_2,\text{Met} \)). The pH profile of \( k_2 \) for hydrolysis of Z-L-Met-ONp showed similar tendency to that for Z-L-Phe-ONp. That is, the hydrolyzing rate was increased with pH and reached the maximum at pH of about 8.

When the relationship between the substrate selective ratio and pH was plotted, however, the ratio exhibits the reverse tendency to that of \( k_2 \) values. That is to say, the substrate-recognizing ability of a glycolipid is found lower due to the rather indiscriminate uptake of substrates, when the catalytic activity is higher or when the uptake of a substrate into vesicles is increased. This agrees well with the fact that the catalytic activity of a glycolipid is more enhanced for a more hydrophilic substrate under a condition fluidized by the addition of 10% phospholipid.

### 3-3 Stability of Vesicles

In order to study the relationship between the fluidity of a vesicle membrane and pH value, the stability of vesicle was examined by leakage of a fluorescent probe included inside the vesicle. Shown in Fig. 7 is the amount of leakage with time. When the probe leaks easily from a water phase within vesicle, this is estimated as the unstable. At the pH over 9, most of a fluorescent probe leaked from the vesicle during the preparation of a vesicle solution. This fact means that the vesicles were more unstable at a Niger pH and the vesicle membrane was more fluidized. The relationship between \( k_2 \) and the stability of vesicles estimated as the initial rate of leakage of a fluorescent probe (Fig. 8) shows that the fluidity of vesicle membrane is higher at the high pH, and suggests that a substrate consequently is taken more easily or faster in the hydrophobic field.
Fig. 7 Stability of Glycolipid Vesicles (in buffer solution, at 25°C).

4 Conclusion

As a conclusion, the pH-dependence of catalytic activity of a glycolipid is illustrated schematically as in Fig. 9. The fluidity of vesicles is lower at a lower pH, because the intermolecular hydrogen bonds are formed among sugar moieties of glycolipids on the vesicle surface. As the dissociation constant pKa of hydroxyl groups of a glycolipid was 6.95 measured by an acid-base titration, such hydrogen bonds were decreased and the amount of an alkoxy anion as active species was increased with increase in hydroxide ion. In addition, the mutual repulsion of alkoxy anions occurs, resulting in more disorder of vesicle membranes and easier uptake of a substrate. This also enhances hydrolysis rate. The catalytic activity of a glycolipid, thus, was increased synergistically with pH. The dissociation of more hydroxyl groups, however, will induce much higher electric repulsion to make vesicles more unstable. As a result, the ability of glycolipids to constitute a molecular assembly is lowered. This phenomenon at a higher pH value, therefore, will decrease the catalytic activity. That is to say, the combination of above-mentioned factors for hydrolysis activity may explain the pH-dependent profile of $k_2$ having the maximum at about pH 8.

The above-mentioned results suggest that the catalytic activity of a glycolipid is explained on the

![Vesicle of glycolipid catalysts](image)

$pK_a = 6.95$

Fig. 9 Explanation for pH-Dependence of Hydrolyzing Activity of Glycolipid Catalyst.
basis of the form of molecular assembly. The relationship between activity and its surroundings (species and amount of phospholipids) of an enzyme must be clarified by further detailed examination of the biomembrane model consisting of the glycolipid and many other kinds of phospholipids.

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References

総説 人工脂質膜を用いた植物ウイルス感染機構の解析

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単分子膜やリポソームの人工脂質膜を利用した植物ウイルス感染初期過程の物理化学的解析について述べた。植物ウイルス外被タンパク質の脂質膜への侵入機構を明らかにできれば感染阻害剤の開発が可能となると考え、まず、強毒ウイルス（タバコモザイクウイルス、TMV）とそのミュータントである弱毒ウイルス（キュウリ緑斑モザイクウイルス、CGMMV）の外被タンパク質の自己合合特性や構造の差異、及び脂質膜への侵入の差異について検討した。その結果、疎水性の高いTMVタンパク質の方が脂質膜の疎水性成分に侵入しやすいことを明らかにした。次に、原形質膜中で存在しウイルスに特異的に結合する感染特異的膜物質を脂質膜中に埋め込んだときには、侵入したウイルスタンパク質の構造変化が小さく、かつ、より脂質膜に取り込まれることが示された。多糖類存在下では原形質膜への脱タンパク化の過程が阻害されるために、感染阻害作用を示すことが明らかとなった。これらの知見に基づく新規な抗ウイルス剤の探索や開発が期待される。

（連絡者：佐野 洋）Vol. 48, No. 6, 543 (1999)

報文 糖脂質酵素モデル（第14報）触媒活性に及ぼすpHの影響

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糖脂質加水分解酵素モデルを種々のpH条件で使用して、Z-フェニルアラニンおよびZ-メチオニンp-ニトロフェニルエステルを加水分解した。糖脂質はα-キモトリプシンに類似の加水分解活性のpH依存性を示し、反応場としてのホスファチジルコリンの有無に拘わらずpH8に頂点を持つ山形のpH依存曲線を示した。また同時に、糖脂質の基質識別能はそのpH依存性と逆の傾向を示し、分解速度が速ければ速いほど識別能の低下することが分かった。このようなpH依存性は、pHが高くなるに従って活性種の量が増加することとベンズル膜の安定性が減少することとの組み合わせによって説明できた。

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