Interaction between Polyoxin and Active Center of Chitin Synthetase†

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Various derivatives of polyoxin C, other polyoxins and several uridine analogues have been known as competitive inhibitors of chitin-UDP acetylglucosaminyltransferase (EC 2.4.1.16, chitin synthetase). Their inhibitory activities were more or less dependent on pH. The variation of inhibitor constants K_i or Michaelis-Menten parameters K_m and V with pH was investigated and the data obtained were plotted according to the method proposed by Dixon *et al.* From the results of the pK_i -pH plots for the above competitive inhibitors, it was concluded that the ionized amino group at C-2'' position acted a very important role for the binding of polyoxins to chitin synthetase. The carbonyl oxygen atoms at C-1'' and of the carbamoyloxy group probably participated in the hydrogen bond formation with the enzyme. And pH scarcely influenced on the interaction between the carboxyl group at C-5' and the enzyme. The results of the Dixon plots for variations of K_m and V with pH suggested that an un-ionized imidazole group ($pK_a=6.3$) and an ionized amino group ($pK_a=7.7$) of chitin synthetase were concerned in the enzyme reaction.

SCHEME 1.

	R ₁	R ₂
N-Acetyl-PoC ^{a)}	-CH ₃	-CH ₂ OH
Glycyl-PoC	-CH ₂ (NH ₂)	-CH ₂ OH
Glycyl-TPoC ^{a)}	-CH ₂ (NH ₂)	-CH ₃
L-Alanyl-TPoC	-CH(NH ₂)CH ₃	-CH ₃
L-Norvalyl-TPoC	-CH(NH ₂)CH ₂ CH ₂ CH ₃	-CH ₃
δ-CAO ^{a)} -DL-norvalyl-TPoC	-CH(NH ₂)CH ₂ CH ₂ CH ₂ OCONH ₂	-CH ₃
Polyoxin L	-CH(NH ₂)CH(OH)CH(OH)CH ₂ OCONH ₂	-H
Polyoxin M	-CH(NH ₂)CH ₂ CH(OH)CH ₂ OCONH ₂	-H

^{a)} The abbreviations used are: PoC, polyoxin C; TPoC, thymine-polyoxin C; CAO, carbamoyloxy.

In our previous papers^{1,2)} the relation of polyoxin structure to chitin synthetase inhibition has been studied in detail by the kinetic investigation using polyoxins and their

analogues, which inhibit the enzyme in competition with the substrate uridine 5'-diphosphate N-acetylglucosamine (UDP-GlcNAc). From the results obtained, it has been concluded that the carbamoyl polyoxamic acid moiety^{*1}

† Studies on the Mode of Action of Polyoxins. Part V. Preceding paper in this series is Ref. 2).

*1 See Scheme 1 and Ref. 3).

($-\text{C}(\text{O})\text{CH}(\text{NH}_2)\text{CH}(\text{OH})\text{CH}(\text{OH})\text{CH}_2\text{OC}(\text{O})\text{NH}_2$) of polyoxins helps to stabilize the polyoxin-enzyme complex by the contributions of its $-\text{C}(\text{O})\text{CH}-$ group ($-\Delta g^{\ast 1}=0.5$), $-\text{NH}_2$ group ($-\Delta g=1.3$) at C-2'', aliphatic carbon chain ($-\Delta g=1.0$), $-\text{OH}$ groups ($-\Delta g=0.9$) at C-3'' and C-4'', and terminal $-\text{OC}(\text{O})\text{NH}_2$ group ($-\Delta g=0.4$). Further, it has been inferred that a specific binding site, to which the uridine moiety of UDP-GlcNAc is bound, lies on the enzyme, and that the pyrimidine nucleoside moiety of polyoxins also fits into this binding site.

It has been reported in the preceding paper²⁾ that the K_i values for polyoxins vary with pH. The investigation on variation of inhibition with pH has been frequently used as a mean for interpretation of action of inhibitors. When an enzymic reaction is carried out in a limited range of pH in which the enzyme protein is stable, the relationship between pH and reaction velocity often shows a bell shaped profile. And from the theory proposed by Dixon and Webb⁴⁾ this result is possible to explain as that specific dissociation groups are contained in the enzyme and the reaction can proceed only in a characteristic state of ionization of these groups. The two proton model for above explanation is summarized in Scheme 2. Where Ke_1 and Ke_2 represent dissociation constants for two groups of a free enzyme HE, and Kes_1 and Kes_2 represent dissociation constants for these groups of an enzyme-substrate complex HES. For determining pKe and $pKes$ values, Dixon *et al.*⁴⁾ have described a simple graphical method in which pH is plotted against pKm ($=-\log Km$), $\log V$ or $\log (V/Km)$. By substituting pKi ($=-\log Ki$) for pKm , the theory described above is applicable to interpret the action of inhibitors.

In the present paper, the results of such a kinetic study using particulate chitin synthetase preparation from *Piricularia oryzae* and its competitive inhibitors are described, and the

SCHEME 2.

interaction between the active center of the enzyme and the inhibitor polyoxin or the substrate UDP-GlcNAc is discussed.

MATERIALS AND METHODS

N-Acetyl-PoC,^{*2,3)} glycyl-PoC,⁵⁾ glycyl-TPoC,^{*2,5)} L-alanyl-TPoC,⁵⁾ L-norvalyl-TPoC,⁵⁾ β -CAO^{*2}-DL-norvalyl-TPoC,⁵⁾ methyl-PoC^{*3} and polyoxin J³⁾ were generous gifts from Dr. K. Isono, the Institute of Physical and Chemical Research. Polyoxins A,³⁾ B,³⁾ D,³⁾ L,³⁾ M,⁶⁾ polyoxin C acid³⁾ and uracil-polyoxin C³⁾ were generously given by Dr. K. Shibuya, Kaken Chemical Co., Ltd. Other chemicals used and preparation of particulate chitin synthetase from *P. oryzae* P₂ have been described in the preceding paper.²⁾ The enzyme fraction obtained was suspended in 1.5 ml of ice cold distilled water and used for experiments immediately. The protein content was about 20 mg per milliliter. The incubation mixture, for assay of the enzyme activity, contained UDP-GlcNAc-¹⁴C (24,000 dpm) at a concentration 7.2~0.7 mM, 20 mM N-acetylglucosamine, 3.2 mM MgCl₂, 0.7 mM mercaptoethanol, 10 μ l of the enzyme solution and 60 μ l of 0.05 M Tris-malate-NaOH buffer with a wide range of pH in a total volume of 100 μ l. Incubation was carried out for 10 min at 25°C and radioactive chitin formed was separated and counted as described previously.²⁾ Michaelis-Menten parameters Km and V , and inhibitor constant Ki were determined by plotting $1/v$ (m μ moles/10 min, 25°C) against $1/S$ (mM UDP-GlcNAc).⁷⁾

RESULTS

Effect of pH on chitin synthetase

The effects of pH on the enzyme activity

*1 Partial binding affinity (kcal/mole, 25°C) has been used to express the contributions of some atoms and atomic groups of polyoxins to the polyoxin-enzyme interaction. See Ref. 2).

*2 The abbreviations used are: PoC, polyoxin C; TPoC, thymine-polyoxin C; CAO, carbamoyloxy.

*3 Methyl ester of the carboxyl group at C-5' of polyoxin C. Unpublished work by Dr. K. Isono.

and on the enzyme stability were investigated over the pH range from 5.2 to 8.6 (Fig. 1). The enzyme activity as a function of pH gave a bell-shaped curve and showed the optimum pH at 7.0. The pH range from 6.0 to 8.6, in which the enzyme was found to be stable, was used in the following experiments.

FIG. 1. Effect of pH on Chitin Synthetase.

For curve A the enzyme activity was assayed for 10 min at pH values given; for curves B, C and D the enzyme was exposed for 5 min, 10 min and 15 min, respectively, to pH values given and the residual activity then tested at pH 7.2. Other conditions for the assay of the activity were the same as described in the text except UDP-GlcNAc concentration was 2.6 mM.

pH dependence of K_m and V

The Michaelis-Menten parameters K_m and V were determined over the pH range 6.0~8.6. The values of pK_m , $\log V$ and $\log (V/K_m)$ were calculated from these values and they were plotted against pH according to Dixon *et al.*⁽⁴⁾ (Fig. 2). Both $\log V$ and $\log (V/K_m)$ as a function of pH showed bell-shaped profiles. As indicated in Fig. 2, the values of pK_{e1} (=6.3), pK_{e2} (=7.7), pK_{es1} (=6.0) and pK_{es2} (=8.0) were determined according to the graphical method of Dixon *et al.*⁽⁴⁾

FIG. 2. Effect of pH on K_m and V for Chitin Synthetase.

The experiment using UDP-GlcNAc as the substrate was carried out as described in the text. The values of K_m and of V obtained were plotted according to Dixon and Webb.⁽⁴⁾

Effect of pH on inhibitions of chitin synthetase by competitive inhibitors

The inhibitory activities of various competitive inhibitors of chitin synthetase as a function of pH were investigated and the results shown in Figs. 3 and 4 were obtained. As Fig. 3 showed, maximum inhibition by polyoxins B, D, J and L occurred at pH 6.8, and above it there was a rapid fall in inhibitory activity. In the case of four N-aminoacyl derivatives of thymine-polyoxin C, the activities were almost constant over pH range 6.0~7.6 and showed a gradual decrease from pH 8.0. The pH dependence of the activity of uracil-polyoxin C and of methyl-polyoxin C was very small. N-Acetyl-PoC gave the sigmoid pH profile which has bends at pH 7.2 and 8.0. As shown in Fig. 4, the pH profiles for uridine and dthymidine apparently differed from those for UMP, UDP and UDP-glucose. The effect of pH on the activity of the former was very small, while the latter was remarkably affected by pH.

FIG. 3. Effect of pH on Chitin Synthetase Inhibition by Polyoxins.

The experiment was carried out as described in the text except that the reaction mixture contained 2.6 mM UDP-GlcNAc and the following compounds at concentrations indicated.

(I).

●—●, polyoxin B 10 μ M; Δ — Δ , polyoxin D 10 μ M; ○—○, polyoxin J 10 μ M; ▲—▲, polyoxin L 10 μ M; ■—■, δ -CAO-DL-norvalyl-TPoC 125 μ M; □—□, L-norvalyl-TPoC 125 μ M.

(II).

○—○, uracil-polyoxin C 3.5 mM; □—□, methyl-PoC 3 mM; ●—●, N-acetyl-PoC 2.5 mM; Δ — Δ , glycyL-TPoC 625 μ M; ▲—▲, L-alanyl-TPoC 250 μ M.

FIG. 4. Effect of pH on Chitin Synthetase Inhibition by Uridine Compounds and *d*Thymidine.

The experiment was carried out as described in Fig. 3. Δ — Δ , uridine 3 mM; ●—●, UMP 3 mM; □—□, UDP 3 mM; ■—■, UDP-glucose 5 mM; ○—○, *d*thymidine 10 mM.

pH dependence of K_i for competitive inhibitors of chitin synthetase

As described above, the inhibitions by several competitive inhibitors were remarkably affected by pH, in contrast the effect of pH on the inhibitions by some other competitive inhibitors was small over pH range given. On the

basis of these results, polyoxins L and M, L-norvalyl-TPoC, glycyL-PoC, N-acetyl-PoC, methyl-PoC, uracil-PoC, uridine and UMP were chosen and inhibitions of the enzyme by them were investigated as a function of UDP-GlcNAc concentration at various pH values. The data obtained were plotted according to Lineweaver-Burk.⁷⁾ These plots showed that they inhibited the enzyme competitively at all pH values tested. The K_i values for the inhibitors at each pH were determined and from these values the pK_i values were calculated and plotted against pH according to Dixon *et al.*⁴⁾ (Figs. 5 and 6). The pH profiles of inhibitory activity and pK_i for an inhibitor were similar each other.

As shown in Fig. 5, the pK_i for polyoxins L and M were found to be approximately independent of pH over pH range 6.0~7.2 and 6.0~7.4, respectively, and then above them there were rapid falls in pK_i values. The pK_i -pH curves for the former and the latter had bends at pH 7.2 and 7.4, respectively. GlycyL-PoC and L-norvalyl-TPoC gave quite similar curves each other, which were roughly parallel to the axis of abscissa over pH range

FIG. 5. Effect of pH on K_i Values for Uracil Polyoxin C, Polyoxins L, M and Derivatives of Polyoxin C. The experiment was carried out as described in the text and the K_i values obtained were plotted against pH according to Dixon and Webb.⁴⁾
 ■—■, polyoxin L; ▲—▲, polyoxin M; ▽—▽, norvalyl TPoC; ○—○, glycyI-PoC; ●—●, N-acetyl-PoC; △—△, methyl-PoC; □—□, uracil-polyoxin C.

FIG. 6. Effect of pH on K_i Values for Uridine and UMP.

K_i for uridine and UMP and K_m for UDP-GlcNAc* are expressed as K . ○—○, uridine; ●—●, UMP; □—□, UDP-GlcNAc.

* The pK_m -pH curve obtained in Fig. 6. is shown here again.

6.0~7.6 and showed bends at pH 8.0. However, the pK_i value for the latter was larger than that for the former by about one unit

at all pH values investigated. N-Acetyl-PoC gave the characteristic pH profile in comparison with its analogues, glycyI-PoC, methyl-PoC and uracil-polyoxin C. The pK_i -pH curve for N-acetyl-PoC showed bends at pH 6.6 and 6.9. The pK_i for this compound was approximately the same as that for glycyI-PoC over pH range 6.0~6.4, then it decreased as pH increased and above pH 7.0 it became roughly close to that for methyl-PoC. The pK_i values for uracil-polyoxin C and methyl-PoC were slightly affected by pH. In Fig. 6, it was found that the pK_i -pH profiles for uridine and UMP have some similarities between the pK_m -pH profile for UDP-GlcNAc over pH range 7.2~8.6. Below pH 7.0, the pK_i for UMP was larger by 0.1~0.3 unit than that for uridine and showed a downward curvature at pH 6.7.

DISCUSSION

The results obtained from the pK_i -pH profiles for uracil-polyoxin C, methyl-polyoxin C and N-acetyl-PoC indicate that the ionized carboxyl group at C-5' influences little on the pH behaviour of N-acetyl-PoC, however that the acetyl group of this compound causes the two bends at pH 6.6 and 6.9 on its pK_i -pH curve. Therefore, it is presumed that the carbonyl oxygen atom of the acetyl group forms a hydrogen bonding with a positively charged group of the enzyme (*e.g.*, the imidazole group of a histidine residue).

It has been described previously^{1,2)} that the amino group at C-2'' position of polyoxins is very important group for the polyoxin-enzyme interaction. The pK_a values of the amino groups of polyoxins L and M have been reported to be 7.0³⁾ and 7.4,⁶⁾ respectively. The results obtained in this paper have indicated that the pK_i -pH profiles for polyoxins L and M have the bends at pH 7.2 and 7.4, respectively, and above these pH values their inhibitory activities decrease rapidly. Each of the pH values of the bends approximately corresponds to the pK_a of the amino group of above polyoxins. Therefore it is possible to say that

the bends observed in the pKi -pH profiles must arise from disappearance of the positive charge of the amino groups at C-2'' of the polyoxins. Although the pH value (=8.0) of the bends found on the pKi -pH curves for glycyl-PoC and L-norvalyl-TPoC is somewhat larger than those pH values found in the cases of polyoxins L and M, these bends may also result from the change in electrical charge of their amino groups at C-2''. When discussion here is confined to pK_a of an amino group, it has been reported³⁾ that the pK_a of deoxypolyoxamic acid is larger than that of polyoxamic acid by 0.3. It has been also known that the pK_a values of several amino acids are larger than those of their hydroxy analogues.¹²⁾ The hydroxyl groups at C-3'' and C-4'' is considered to lower pK_a of the amino group at C-2'', so that the difference in the pH values for the bends described above is found. In any event, the ionized amino group at C-2'' of polyoxins seems to contribute to form a bonding with an appropriate group, perhaps charged negatively, of the enzyme. And, as described previously,²⁾ since the exact position of this amino group is important for the binding of polyoxins to the enzyme, the above assumed group of the enzyme is seemed to have a specific position corresponding to the amino group.

The pH profile of the inhibitory activity of δ -CAO-DL-norvalyl-TPoC shows more rapid fall with increasing of pH than that for L-norvalyl-TPoC over pH range 7.2~8.2. δ -CAO-DL-norvalyl-TPoC is an equivalent mixture contains each isomer at a concentration of 62.5 μ g/ml. At this concentration, the D-isomer scarcely affects the enzyme.²⁾ Therefore, it is suggested that the difference found in the two pH profiles presumably arises from the presence of carbamoyloxy group. The carbonyl oxygen atom of this group may participate in the hydrogen bond formation with an appropriate group, perhaps charged positively, of the enzyme.

The pKi -pH profile for uridine and for UMP have bends at pH 7.8 and 8.0, respectively. Neither of these compounds contain any dis-

sociation group whose pK_a is above the values. Therefore, the bends are possibly attributed to a change in electrical charge of a dissociation group (*e.g.*, an amino group) of the free enzyme and of the enzyme-inhibitor complex. Uridine, UMP and UDP-glucose competitively inhibit the enzyme, while little inhibition is observed by cytidine, CMP and CDP-glucose, which structures are very similar to their uridine counterparts, except oxygen atom at C-4 position of uracil is substituted by amino group.^{1,2)} These findings suggest that this amino group interferes with the interaction between the cytidine analogues and the binding site. Conversely, it is presumed that the oxygen atom at C-4 participates in the uridine-binding site interaction. From the pKm -pH profile for UDP-GlcNAc, the pK_{e2} and the pK_{e3} are found to be 7.7 and 8.0, respectively. An amino group is suppose as a dissociation group which shows such pK_{e2} value. It is likely that a positively charged amino group of the binding site is concerned in the formation of hydrogen bonding with an appropriate atom, presumably the oxygen atom at C-4, of the uridine moiety of UDP-GlcNAc. Thus the affinity of uridine to the enzyme is weakened with disappearance of the positive charge. The $-\Delta g$ for the hydroxyl group at C-2' of the ribose moiety is approximately 1.0 kcal/mol.²⁾ This and another hydroxyl group at C-3' probably contribute to form the hydrogen bonding with relevant groups of the enzyme.

It has been described previously²⁾ that the $-\Delta g$ for the phosphate group of UMP and for the pyrophosphate group of UDP are 0.1 and 0.2 kcal/mol, respectively. The pKi -pH curve for UMP shows, as compared with that for uridine, that the phosphate group is effective on the Ki of this compound over pH range 6.0~7.0, and indicates the downward curvature near pH 6.7. While the pK_{e1} and the pK_{e3} found are about 6.3 and 6.0, respectively. The imidazole group of a histidine residue has been known as a dissociation group having such pK_{e1} value.⁴⁾ And the imidazole group has been proposed as an essential part of the active site of various enzyme⁸⁾ such like α -

chymotrypsin,⁹⁾ fumarase¹⁰⁾ and acetylcholinesterase.¹¹⁾ From these facts, it is concluded that an unprotonated imidazole group presumably interacts with the pyrophosphate moiety of UDP-GlcNAc in the catalytic process of the enzyme. In the case of UMP, its free phosphate group may be bound to the same but positively charged imidazole group. That is to say, the former action is nucleophilic and the latter action is electrophilic.

The proposed mechanism of interaction

FIG. 7. Proposed Mechanism of Interaction between Polyoxin (I) or UDP-N-acetylglucosamine (II) and Active Center of Chitin Synthetase.

* The values of partial binding affinity for the $-\text{COOH}$ and the $-\text{R}(=\text{CH}_3)$ groups are 0.7 and 0.4 kcal/mole, respectively.²⁾

between polyoxin or UDP-GlcNAc and the active center of chitin synthetase is summarized in Fig. 7.

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