

EFFECT OF pH ON THE FORMATION OF INCLUSION COMPLEXES BETWEEN β -LACTAM ANTIBIOTICS AND 2-HYDROXYPROPYL- β -CYCLODEXTRIN IN AQUEOUS SOLUTION

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The complexation of β -lactam antibiotics, amoxicillin (AMPC), ampicillin (ABPC) and benzylpenicillin (PCG), with 2-hydroxypropyl- β -cyclodextrin (HPCD) was studied at various pH values using microcalorimetry, ^1H NMR spectroscopy, and molecular dynamic simulation. In the strong acid solution, two different types of inclusion complex with a 1:1 stoichiometry, Complex I with a phenyl ring of β -lactam antibiotics penetrated into the cavity of HPCD and Complex II with a penam included in the cavity, were formed by hydrophobic interaction, and Complex II was more stable than Complex I. In aqueous solution at $\text{pH} \geq 4.5$, only Complex I was formed, where the penam of PCG was more deeply penetrated into the cavity to keep it stable than those of AMPC and ABPC. The charged carboxyl-group on the penam was less affinity to form Complex II.

Keywords: amoxicillin, ampicillin, benzylpenicillin, 2-hydroxypropyl- β -cyclodextrin, inclusion complex, microcalorimetry, molecular dynamic simulation

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of α -1,4-linked D-glucopyranose units. The most common of these ring-shaped molecules are the α CD, β CD and γ CD formed by six, seven and eight glucose units, respectively. 2-hydroxypropyl- β -cyclodextrin (HPCD) used in this study is a modified β CD to be readily soluble in water and a pharmaceutically useful CD derivative. CDs form the non-covalently bonded inclusion complex with a variety of drugs as guest molecule in both solution and solid state. In the pharmaceutical field this complexation phenomenon has been extensively applied to enhance the solubility, dissolution rate, and bioavailability of sparingly soluble drugs in gastrointestinal fluids [1–3].

Amphoteric penicillins like ampicillin (ABPC) and amoxicillin (AMPC) exhibit a broad spectrum of antibacterial activity, are widely used in the treatment of a variety of infectious diseases. ABPC and AMPC differ from benzylpenicillin (PCG) only by the presence of an amino group (Fig. 1), which helps the drug penetrate the outer membrane of gram-negative bacteria. However, these β -lactam antibiotics are commonly susceptible to degradation by hydrolysis and β -lactamase-producing bacteria. The degradation in gastric juice decreases bioavailability in human subjects and the polymers formed by degradation products possess strong antigenic properties. We have

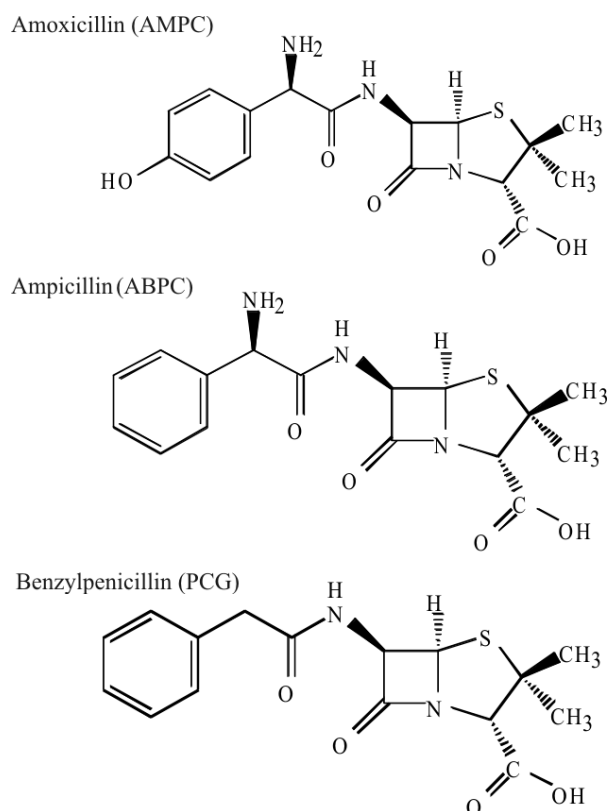


Fig. 1 Structures of β -lactam antibiotics. Amoxicillin (AMPC); $\text{pK}_{\text{a}1}=2.6$, $\text{pK}_{\text{a}2}=7.3$ and $\text{pK}_{\text{a}3}=9.7$; Ampicillin (ABPC); $\text{pK}_{\text{a}1}=2.5$ and $\text{pK}_{\text{a}2}=7.5$ and benzylpenicillin (PCG); $\text{pK}_{\text{a}}=2.76$

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reported that the degradation and the polymerization of ABPC in acidic solution were inhibited by complexation with β CD and HPCD, and that the antibacterial activity of AMPC to *Escherichia coli B* became stronger in the following order: only AMPC < AMPC- α CD complex < AMPC- γ CD complex < AMPC- β CD complex [4–6].

Since ABPC and AMPC exist as a cation, a zwitterion, or an anion due to pKa values, the formation of inclusion complex would be influenced by pH in a solution. In our previous reports [4–7], it was indicated that the cationic ABPC and AMPC formed two types of inclusion complex with a 1:1 stoichiometry and also a 1:2 (guest molecule: β CD) complex. Therefore, the aim of this work was to clarify the pH-dependent structures, stoichiometries, and the stability behavior of the inclusion complex between β -lactam antibiotics (AMPC, ABPC and PCG) and HPCD using isothermal microcalorimetry, NMR spectrometry, and molecular dynamic simulation. On the basis of the thermodynamic parameters obtained from structurally related guest, the mechanisms and thermodynamic origin for the formation of inclusion complex in aqueous solution would be established.

Experimental

Materials

Amoxicillin anhydride (AMPC), ampicillin (ABPC) and deuterated solvents (D_2O , DCl and NaOD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2-Hydroxypropyl- β -cyclodextrin (HPCD; FW=1540, DS=1.0) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Benzylpenicillin (PCG), β -cyclodextrin (β CD) and other materials of analytical reagent grade were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Clark-Lubs solution (1/5M HCl, 1/5M KCl and H_2O) at pH 1.2 and 2.0, and Sørensen solution (1/15M KH_2PO_4 and 1/15M Na_2HPO_4) at pH 4.5, 6.0 and 8.5 were used as buffer solutions. All the chemicals used in this study were of analytical reagent grade.

Instrumental methods

NMR spectrometry

1H NMR spectra of AMPC were taken on JEOL GX-400 interfaced with a DEC RSX-11M computer operating at 298 K. The concentration of AMPC was kept constant at $1.4 \cdot 10^{-4}$ M. The mixture of AMPC and HPCD at various HPCD/AMPC molar ratios in D_2O solutions following adjustment of

pD values to 1.0, 6.0 and 8.0 with 37% DCl and 40% NaOD were prepared. The chemical shifts for free (δ_{free}) and complex ($\delta_{complex}$) of AMPC were measured in the absence and presence of HPCD, respectively, and assigned based on the external standard sodium 2,2,3,3-d₄-3-trimethylsilylpropionate. Induced change in each chemical shift of AMPC ($\Delta\delta$) by complexation was calculated using the following equation:

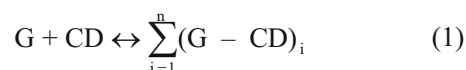
$$\Delta\delta = \delta_{complex} - \delta_{free}$$

Two-dimensional nuclear overhauser enhancement spectra (NOESY) was measured for the mixture with 1:1 molar ratio of β CD/AMPC in 0.1M DCl and D_2O solutions.

Isothermal titration microcalorimetry

Microcalorimetric study was performed with a Thermal Activity Monitor 2277 system (Thermometric, Järfälla, Sweden) at 298.15 ± 0.001 K. Guest molecules (AMPC, ABPC and PCG) and HPCD were dissolved separately in buffer solutions at pH 1.2, 2.0, 4.5, 6.0 and 8.0. A reaction cell was initially filled with a 3.0 mL of each guest solution ($5.0 \cdot 10^{-4}$ M). The heat of reaction was measured by injecting HPCD solution ($2.5 \cdot 10^{-2}$ M) in 15–20 portion of 15 μ L into the cell to a final concentration of $1.25 \sim 20.0 \cdot 10^{-4}$ M. The heat of dilution of HPCD was measured for each sample and subtracted from the heat of reaction.

Calorimetric data were analyzed for a one-step reaction, assuming that a guest molecule (G) and a cyclodextrin (CD) form several types (structures) of inclusion complexes with a 1:1 stoichiometry (G-CD) as follows:



In the case of $i=1$, CD forms only one type of inclusion complex with G. When i is more than 2, several types of inclusion complex are formed independently. The heat effect (ΔQ) of the complexation is proportional to the quantity of complexes.

$$\Delta Q = \Delta H_1(G - CD)_1 + \dots + \Delta H_i(G - CD)_i + \dots + \Delta H_n(G - CD)_n \quad (2)$$

where ΔH_i is enthalpy change for the i th type of inclusion complex (G-CD) _{i} . Assuming that two types of inclusion complex are formed, ΔQ can be expressed as a function of the free concentration of CD ($[CD]$) and the total concentration of guest molecule ($[G]_t$).

$$\Delta Q = [G]_t V \left(\frac{\Delta H_1 K_1 [CD]}{1 + K_1 [CD]} + \frac{\Delta H_2 K_2 [CD]}{1 + K_2 [CD]} \right) \quad (3)$$

where K_1 and K_2 represent association constants of the complexes $(G-CD)_1$ and $(G-CD)_2$, respectively, and V is volume in a reaction cell.

The best fit values of the association constants and the enthalpy changes in one- and two-step reactions can be estimated using an iterative non-linear least squares curve-fitting program [5].

Molecular dynamic simulation

Molecular dynamic simulation (MDS) of the inclusion complexes between AMPC and HPCD in aqueous solution was performed using the AMBER program (ver. 6) running. As a model compound of HPCD, the modified β -CD in which hydrogen atoms of all the primary hydroxyls were replaced with 2-hydroxypropyl groups was used in this study. For AMPC molecule, the cationic, zwitterionic and anionic species were simulated to form the inclusion complexes with HPCD. The geometries and EPS charges of AMPC and HPCD were optimized using Gaussian programs. In the most realistic model of the complex in aqueous solution, AMPC was manually docked in the HPCD cavity according to the NMR observations to obtain a good starting geometry for the complex. An inclusion complex was placed in the center of a 2.7 nm cubic box containing ca. 500 TIP3P water molecules and subjected to energy minimization to obtain more realistic, low-energy minimization starting structures of MDS using Monte Carlo technique. MDS was equilibrated by 200 ps ($\Delta t=0.001$ ps and 200000 steps) with SHAKE constrains for hydrogen atoms under conditions of constant pressure (0.1 MPa) and temperature (293 K). Intermediated structures were saved in a file very 50 steps to obtain representative, sequential structures generated during the simulation. The time of 200 ps was enough for the system to reach an equilibrium state and the stable structures of complexes were maintained for a long time.

Results and discussion

1H NMR evidence of inclusions

The 1H NMR signals of AMPC alone (δ_{free}) were assigned in D_2O solutions at pD 1.0, 6.0 and 8.0, and it was confirmed that neither degradation of AMPC nor formation of the dimmers caused in these solutions. The induced change, $\Delta\delta$, is defined as the difference of chemical shifts in the presence and absence of HPCD. Figure 2 shows $\Delta\delta$ in the chemical shifts of AMPC as a function of HPCD/AMPC molar ratio. In this convention, a positive sign of $\Delta\delta$ means a shift change to down-field and a negative sign means

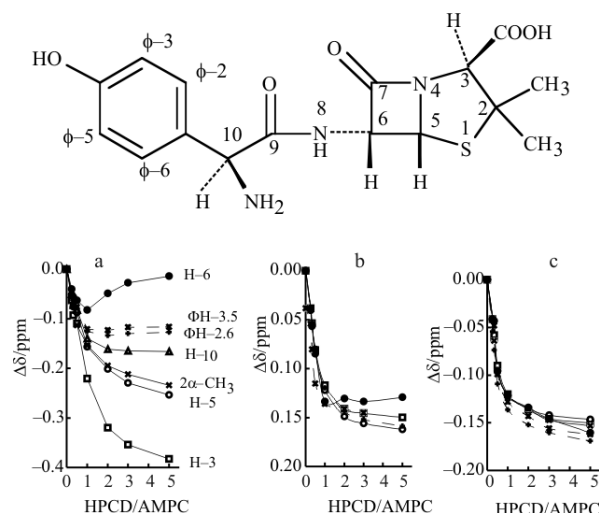


Fig. 2 Changes in 1H NMR chemical shifts of AMPC caused by the complexation between AMPC and HPCD at a – pD=1.0, b – 6.0 and c – 8.0 as a function of the molar ratio of HPCD/AMPC

a shift change to up-field. At pD 1.0, the almost chemical shifts of AMPC moved upfield on the addition of HPCD, and the variation of $\Delta\delta$ for H-3, H-5 and 2α -CH₃ on the penam especially increased as the molar ratio of HPCD/AMPC increased (Fig. 2a). High up-field shifts have generally been noted for complexation [8], suggesting that the penam of AMPC is entrapped by deep penetration in the hydrophobic cavity. However, the changes in $\Delta\delta$ of the penam protons were reduced at pD 6.0 and pD 8.0 as shown in Figs 2b and c, respectively. Slight changes to up-field are probably due to a variation in local polarity when the protons are shielded in the CD cavity [9] and indicates weaker interactions with hydrogen atoms (shielding effect due to van der Waals forces between the guest molecule and carbohydrate chains of CD molecule). The chemical shifts of the protons on the phenyl ring also moved up-field slightly. The shielding effect of the phenyl ring was due to interaction with the ring current as a consequence of complete inclusion within the CD cavity. The largest magnitude of $\Delta\delta$ for the phenyl ring protons was observed at pD 8.0 (Fig. 2c). The same tendency was shown in the chemical shifts for ABPC-HPCD and ABPC- β CD complexation [7].

In a NOESY spectrum of AMPC- β CD complex in 0.1M DCl solution (not shown in this paper), the intermolecular NOEs between the penam protons of AMPC and the protons situated internal cavity of β CD were observed. A lower threshold representation allows the observation of intermolecular NOEs between the phenyl ring protons and the protons of β CD. On the other hand, only one cross peak between the phenyl ring protons of AMPC and β CD protons

was observed in D₂O solution (not shown in this paper).

The results of NMR spectroscopy agreed with the previous our reports that the cationic ABPC and AMPC formed two types of inclusion complex [6, 7]. Furthermore, it was clarified that the affinity of the penam toward the CD cavity was higher than that of phenyl ring in the strong acidic solution, and that the degree of the affinity became lower with increasing pH in aqueous solutions.

Thermodynamic parameters for the complexation between β -lactam antibiotics and HPCD

In this study, the heat effect (ΔQ) for the complexation between PCG and HPCD was measured to obtain the thermodynamic parameters. As same as the previous study for ABPC and AMPC [4, 6], ΔQ increased exothermically as the HPCD concentration increased, but was significantly influenced by pH of the aqueous solution. In analyses of the calorimetric data of ABPC-HPCD and AMPC-HPCD complexation [5, 7], ΔQ at pH 1.2 and 2.0 could be only fitted to the two-type system ($i=2$) for the inclusion complex, while ΔQ at pH 4.5, 6.0 and 8.0 fitted to the both one-type ($i=1$) and two-type ($i=2$) systems in Eq. (2). So, the calorimetric data for PCG-HPCD complexation were analyzed by the two-type system ($i=2$), where two kinds of inclusion complex, the first type with high affinity to HPCD (K_1) and the second type with low affinity (K_2), were formed at same time. The first and the second types in the inclusion complex formed at various pH values were determined based on the results of NMR study.

All the thermodynamic parameters for AMPC, ABPC and PCG at various pH were summarized in Fig. 3. The parameters of ΔG_ϕ , ΔH_ϕ and ΔS_ϕ were related to the complex with the phenyl ring inserted in the CD cavity (Complex I), and ΔG_{PE} , ΔH_{PE} and ΔS_{PE} were to the complex with the penam included in the cavity (Complex II). The complexation was entropy-driven (ΔS_ϕ and $\Delta S_{PE} > 0$), reflecting the hydrophobic interaction between guest molecule and the hydrophobic cavity of HPCD. The values of ΔG_ϕ were kept constant in whole pH values: $\Delta G_\phi = -17.2 \pm 0.3$, -17.6 ± 0.5 and -18.7 ± 0.3 kJ mol⁻¹ for AMPC, ABPC, and PCG, respectively. It was indicated that Complex I was formed in aqueous solution independently of pH. In Complex II, the magnitude of ΔG_{PE} was largest at pH 1.2, where the carboxyl-group of penam was entirely plotted (uncharged), and then decreased. The association constants were reduced by half at pH 2.0 [5, 7], where about 50% of carboxyl-group was charged. The significant reduce of ΔG_{PE} observed from pH 2.0 to 4.5 was attributed to the

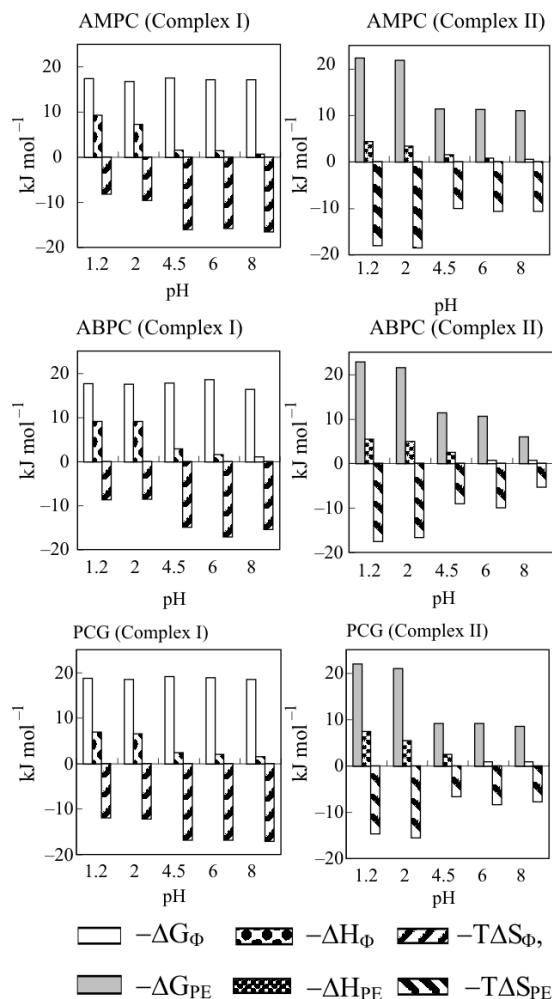


Fig. 3 Thermodynamic parameters of Complex I and II for the complexation between β -lactam antibiotics and HPCD

increment of the charged carboxyl-group. Up to pH value 4.5 the penam with the fully charged carboxyl-group provided very little affinity towards the hydrophobic cavity, and then the association constants were too small (< 100 M⁻¹) to stabilize Complex II.

From a structural point of view, these guest molecules would be able to form a 1:2 (guest:HPCD) complex. The calorimetric data at pH 1.2 and 2.0 were further analyzed using a two-step reaction and the thermodynamic parameters were obtained [5, 7]. On measuring the FAB mass spectrum of the complex, a high intensity peak of the 1:1 complex and a far small intensity peak of the 1:2 complex were observed.

Enthalpy-entropy compensation effect for the complexation

A calorimetric technique is useful not only to evaluate the association constant, but also to determine the enthalpy and entropy changes for complexation. However, it cannot provide a clear answer about the

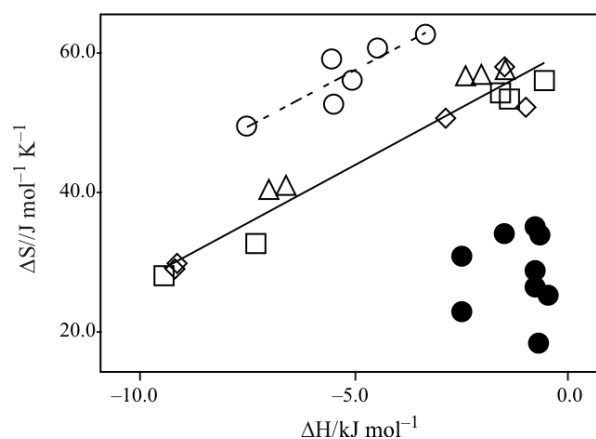


Fig. 4 Enthalpy-entropy compensation plot for the inclusion complexation of β -lactam antibiotics with HPCD. Symbols of \square , \diamond and \triangle represent the thermodynamic parameters (ΔS_ϕ and ΔH_ϕ) derived from Complex I of AMPC, ABPC and PCG. The symbols of \circ and \bullet were the thermodynamic parameters (ΔS_{PE} and ΔH_{PE}) derived from Complex II at $\text{pH} \leq 2.0$ and at $\text{pH} \geq 4.5$, respectively. The solid and the broken lines are computed by linear regression analysis for the $\Delta S_\phi - \Delta H_\phi$ plots and the $\Delta S_{PE} - \Delta H_{PE}$ plots at $\text{pH} \leq 2.0$, respectively

complex mode (inclusion or binding) nor the structural information. So the complex conformations for the first- and the second-type were determined on the basis of $\Delta\delta$ of the guest molecule and NOEs of the complex to determine the thermodynamic parameters for Complex I and II at various pH values. And next, the enthalpy-entropy compensation analysis was performed to confirm the pH-dependent structures of the inclusion complexes [10].

The entropy changes were plotted against the enthalpy changes using all the thermodynamic data obtained from the complexation of AMPC, ABPC and PCG with HPCD (Fig. 4). The plots were divided into three classes: two groups with independently linear relations and one group with non-linear relation. The $\Delta S_\phi - \Delta H_\phi$ plots for Complex I lay on a straight line with a correlation coefficient of 0.97. In the formation of Complex II, the $\Delta S_{PE} - \Delta H_{PE}$ plots at pH 1.2 and 2.0 also showed a linear relationship with a correlation coefficient of 0.93. The complexation phenomenon can be understood in a general context of the enthalpy- entropy compensation effect as far as the weak interactions through hydrophobic interactions, van der Waals forces, and hydrogen bonds, and the isoequilibrium temperature can be estimated from the slope of a straight line [11, 12]. The values of 294 and 302 K calculated from line A and line B, respectively, were approximately equal to the experimental temperature (298 K). The empirical linear relationship between ΔS_ϕ and ΔH_ϕ means that Complex I is constantly formed in aqueous solution at

whole pH range. The values of ΔS_ϕ for PCG-HPCD complexation were positively larger than those of ABPC-HPCD and AMPC-HPCD complexation, suggesting that the phenyl ring of PCG more deeply penetrated into the CD cavity. And also the values of ΔS_{PE} at $\text{pH} \leq 2.0$ were much larger than those of ΔS_ϕ , indicating that penam with uncharged carboxyl-group was easily included in the CD cavity by hydrophobic interaction. However, no relation was shown in the $\Delta S_{PE} - \Delta H_{PE}$ plots at $\text{pH} \geq 4.5$, since the values of ΔG_{PE} were too small to form Complex II in the aqueous solution (Fig. 3).

Molecular dynamic simulation (MDS) of AMPC-HPCD inclusion complexes

In MDS, HPCD in which all the primary hydroxyls were substituted with 2-hydroxypropyl groups was used as a host molecule to include AMPC as a guest molecule. Two initial geometries of inclusion complexes (Complex I and II) were considered from the volume of the cavity of HPCD and the bulkiness of AMPC. The phenyl ring was placed in the cavity and the penam was situated outside the wide rim of the cavity as Complex I. The penam was located in the center of the cavity, and the phenyl ring was outside as Complex II. Since AMPC has three pKa values, $\text{pKa}_1=2.54$ ($-\text{COOH}$), $\text{pKa}_2=6.42$ ($-\text{NH}_2$) and $\text{pKa}_3=9.86$ ($-\text{OH}$), different species of cation, zwitterion, anion and di-anion exist dependently on pH. The inclusion behaviors for three species (cation, zwitterion and anion) in aqueous solution was simulated for 200 ps using the AMBER program and reached to equilibrium by 200 ps. The snapshots of the inclusion complexes of at 200 ps were shown in Fig. 5.

All species of AMPC maintained the initial structures of Complex I for 200 ps to be stable with CH/ π interaction between the hydrophobic C-H bond in the cavity and the phenyl ring inserted into the

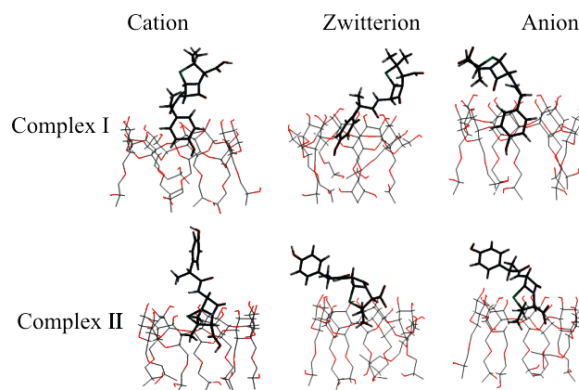


Fig. 5 Snapshots of two types of inclusion complex for cation, zwitterion and anion species of AMPC at 200 ps. See text for an explanation of Complex I and II

cavity. From the results of ^1H NMR spectroscopy, the chemical shifts of the phenyl ring protons moved slightly up-field ($\Delta\delta \approx -0.15$) in all pH values (Fig. 2). The ammonium group ($-\text{NH}_3^+$) in the cationic and the zwitterionic AMPC existed in the neighborhood of the entrance of the cavity and was capable of hydrogen bonding formation and/or electrostatic interaction with hydroxyl groups of CD.

Only the cationic AMPC formed the stable Complex II, since the carboxyl-group on penam was uncharged and hydrophobic to easily insert into the hydrophobic cavity. The results of ^1H NMR spectroscopy presented that chemical shifts of H-3, H-5 and $2\alpha\text{-CH}_3$ on the penam ring moved largely up-field at pD 1.0 (Fig. 2a). In the zwitterion and anion, Complex II was gradually driven out from the cavity. The charged carboxyl-group on penam was located near to the rim. These results indicate that only the cation forms two types of the stable inclusion complex with a 1:1 molar ratio.

Conclusions

All of β -lactam antibiotics used in this study formed two types of inclusion complex with HPCD at 1:1 molar ratio, Complex I with the phenyl ring included in the CD cavity and Complex II with the penam included in cavity, and the formation mechanism or the stability of the complex depended on the pH value of aqueous solution. All of the complexation were entropy-driven due to hydrophobic interaction. Complex I and II were coexisted in the strong acid solution, however, only Complex I was realized in the weak acid, neutral and alkaline solutions. The uncharged carboxyl-group of the penam was necessary to form Complex II. Since the carboxyl-group on the penam is completely charged at $\text{pH} \geq 4.5$, the penam would be less affinity to the hydrophobic cavity of

CD. On the contrary, the charged amino-group in the side chain of AMPC and ABPC seemed to contribute to the stability of Complex I by hydrogen bonding formation and/or electrostatic interaction with the hydroxyl-groups of CD. The phenyl ring without amino-group for CD was deeply penetrated into the CD cavity to stabilize the inclusion complex at various pH of the solution.

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