DOI 10.5012/bkcs.2010.31.02.442

Complex Formation of Adenosine 3',5'-Cyclic Monophosphate with β-Cyclodextrin: Kinetics and Mechanism by Ultrasonic Relaxation

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Received November 12, 2009, Accepted January 25, 2010

Adenosine 3',5'-cyclic monophosphate (cAMP) is a second messenger responsible for a multitude of cellular responses. In this study, we utilized β-cyclodextrin (β-CD) as an artificial receptor with a hydrophobic cavity to elucidate the inclusion kinetics of cAMP in a hydrophobic environment using the ultrasonic relaxation method. The results revealed that the interaction of cAMP with β-CD followed a single relaxation curve as a result of host-guest interactions. The inclusion of cAMP into the β-CD cavity was found to be a diffusion-controlled reaction. The dissociation of cAMP from the β-CD cavity was slower than that of adenosine 5'-monophosphate (AMP). The syn and anti glycosyl conformations of adenine nucleotides are considered to play an important role in formation of the inclusion complex. Taken together, our findings indicate that hydrophobic interactions are involved in the inclusion complex formation of cAMP with β-CD and provide insight into the interactions of cAMP with cAMP-binding proteins.

Key Words: Cyclodextrin, Cyclic nucleotide, Host-guest interaction, Ultrasonic wave

Introduction

Adenosine 3',5'-cyclic monophosphate (cAMP) is an important second messenger in cells produced by adenylyl cyclase upon hormone binding to cell surface receptors.[1] cAMP binds to and regulates a number of proteins including protein kinase A, cAMP-regulated ion channels and Epac (exchange protein directly activated by cAMP) for a myriad of cellular responses.[2] cAMP is then degraded to the corresponding monophosphate 5'-AMP (AMP) by the enzyme cyclic nucleotide phosphodiesterase (PDE) to turn off hormonal signals.[1]

Previous evaluation of the crystallization of PDE revealed that cAMP is bound to the cAMP-binding pocket of PDE *via* an adenine base that is held tightly in the active site by a hydrophobic clamp, but that hydrogen bonds and metal binding also play an important role in formation of the complex.[3,4] In this study, we utilized cyclodextrin (CD), which is composed of glucopyranose units linked by an α-(1 → 4) glucosidic bond, as an artificial receptor with a hydrophobic cavity to elucidate the kinetic properties of cAMP in hydrophobic environments. Three types of CDs occur naturally with 6, 7, and 8 glucopyranose units and these CDs are generally denoted as α-, β-, and γ-CD, respectively.[5] CDs are soluble in water due to their hydrophilic outer surface and are able to form inclusion complexes due to their hydrophobic inner cavity with a variety of guest molecules containing hydrophobic functional groups.[5] The inclusion of guest molecules into the cavity of CD is considered to be a diffusion-controlled reaction,[6-8] while van der Waals forces and hydrophobic interactions are primarily responsible for the formation of the inclusion complex.[9] The dissociation, however, is dependent on the structure of the guest molecules.[10]

A number of techniques have been utilized to elucidate the molecular interactions that occur between β-CD and AMP including chromatography,[11] circular dichroism spectroscopy,[12,13] and ultrasonic relaxation;[8,14] however, inclusion complex formation of cAMP with β-CD has not yet been explored. Therefore, we sought to elucidate the inclusion kinetics of cAMP into the β-CD cavity using the ultrasonic relaxation method in the frequency range of 0.2 ~ 50 MHz.

Experimental Methods

Materials. β-CD and cAMP sodium salt were purchased from Sigma (St. Louis, MO). Sample solutions with pH values around 6.9 were freshly prepared prior to each experiment. All experiments were conducted at 25 °C. The solution densities were measured using a vibrating density meter (Anton Paar DMA 5000M).

Ultrasonic Relaxation Measurements. The following three experimental techniques were used to measure ultrasonic absorption covering a wide frequency range of 0.2 ~ 40 MHz: a plano-concave resonance method (0.2 ~ 2 MHz), a plano-plano resonance method (3 ~ 8 MHz), and a beam reflection method (25 ~ 50 MHz). The velocity was measured using a pulse-echo method at 3 MHz. The key apparatus used in this study employed a high-Q ultrasonic resonance method equipped for the lower frequency range. Briefly, standing waves were established in a cylindrical cavity, composed of a 2-MHz fundamental X-cut quartz transducer and a concave reflector. The diameter of the cavity was 56 mm and the sample volume size was 50 cm^3. Using the Raman-Nath light diffraction method, a resonance spectrum was obtained with an optical heterodyne detection system. This technique allows the half-bandwidth of one resonance curve to give the absorption coefficient of the sample liquid. The high-quality factor attained with this resonator cell allowed reliable absorption measurements below 1 MHz. The instrumental loss above 300 kHz was negligible and the loss

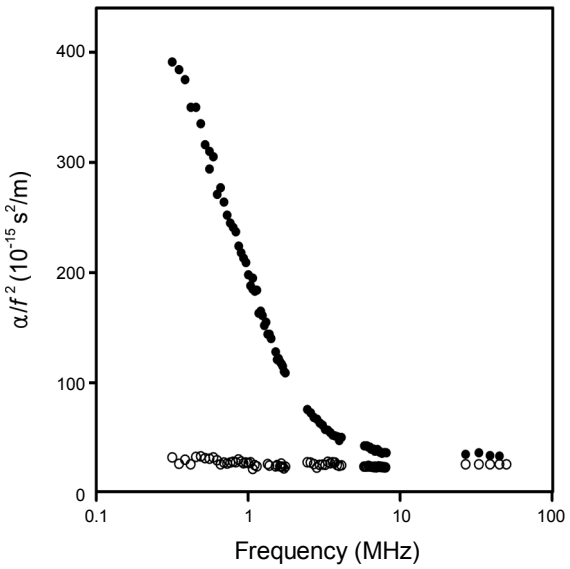

Figure 1. Ultrasonic absorption α/f^2 *vs.* frequency in aqueous solution of cAMP at 25 °C in the presence and absence of 8.7 mM β-CD: (○) 10 mM cAMP only, (●) 7 mM cAMP + 8.7 mM β-CD.

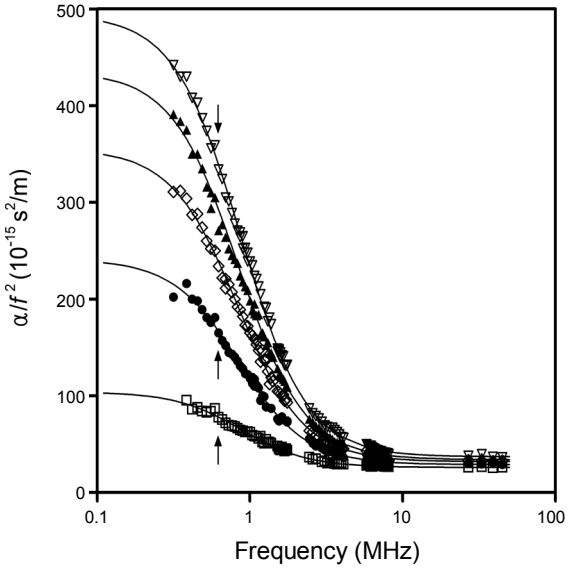

Figure 2. Excess absorption spectra of cAMP in aqueous solutions in the presence of 8.7 mM β-CD at 25 °C. (□) 1 mM cAMP + 8.7 mM β-CD; (●) 3 mM cAMP + 8.7 mM β-CD; (◇) 5 mM cAMP + 8.7 mM β-CD; (▲) 7 mM cAMP + 8.7 mM β-CD; (▽) 9 mM cAMP + 8.7 mM β-CD. The solid lines represent the values calculated from equation (1). The arrows indicate the location of the relaxation frequency.

below 300 kHz was calibrated using water. A plane-plano resonator cell consisting of 5-MHz fundamental X-cut quartz crystals, 2 cm in diameter, was used for the frequency range between 3 and 8 MHz.

Results

To investigate the ultrasonic relaxation of β-CD and cAMP, we first measured the relaxations of cAMP in aqueous solution. Although the syn and anti conformations of cAMP as a result

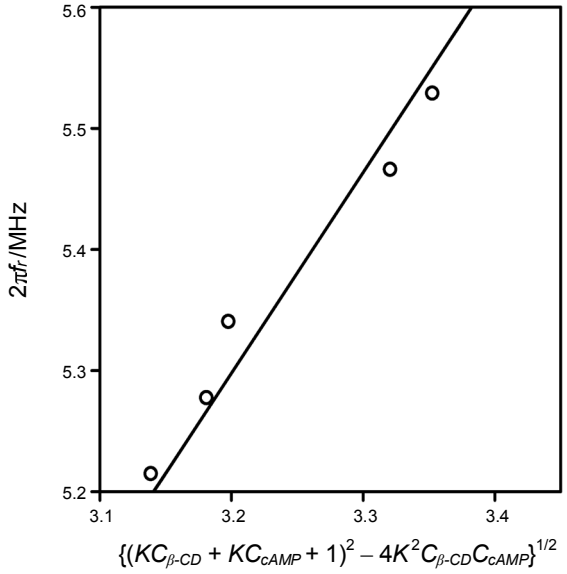

Figure 3. Plots of $2\pi f_r$ *vs.* $\{(KC_{\beta\text{-}CD} + KC_{cAMP} + 1)^2 - 4K^2C_{\beta\text{-}CD}C_{cAMP}\}^{1/2}$ for aqueous solution of cAMP in the presence of 8.7 mM β-CD at 25 °C.

of ultrasonic relaxation were reported at concentrations of cAMP above 50 mM,[15] no relaxation was observed when 10 mM cAMP in the frequency range of 0.1 ~ 50 MHz was used (see Figure 1). The ultrasonic absorption coefficient divided by the square of the sound frequency, α/f^2, approached the value of $\alpha/f^2 = 26 \times 10^{-15}$ s^2/m. Since β-CD itself exhibited ultrasonic relaxation at concentrations greater than 13 mM,[16] we fixed the concentration of β-CD at 8.7 mM. Although no excess absorption was observed in individual solutions of β-CD and cAMP, the values of α/f^2 were dependent on the frequency when the solution had both β-CD and cAMP.

The ultrasonic relaxation represents a chemical equilibrium associated with the inclusion complex formation between β-CD and cAMP. To further examine the relaxation process, we changed the concentration of cAMP from 1 to 9 mM while fixing the β-CD concentration at 8.7 mM. The frequency dependence of α/f^2 was observed in all of the mixed aqueous solutions and therefore it was tested to determine if the values of α/f^2 fit to the usual Debye-type single relaxation equation:

$$\alpha/f^2 = A/\{1 + (f/f_r)\}^2 + B \quad (1)$$

where f_r is the relaxation frequency, and A and B are constants. The ultrasonic parameters, f_r, A, and B, were determined to obtain the fit of the experimental data to Equation (1) using the non-linear least mean square method. The lines drawn through the data points in Figures 1, 2 and 3 were well fitted with the values according to Equation 1. The good agreement between the calculated line and the experimental data confirms that a single relaxation process is involved in the formation of the inclusion complex. The ultrasonic parameters determined in this study are listed in Table 1 along with the values of the sound velocity, υ, and density, ρ.

Since the relaxation appeared only when the two solutes were resolved in water, the cause of the observed relaxation is

Table 1. Ultrasonic Relaxation and Thermodynamic Parameters of cAMP and β-CD in Aqueous Solution at 25 °C

$C_{\beta\text{-}CD}$	C_{cAMP}	f_r	A	B	ρ	υ
mM	mM	MHz	10^{-15} s^2m^{-1}	10^{-15} s^2m^{-1}	kgm^{-3}	ms^{-1}
8.7	1	0.88	78	26	1.0006630	1499.50
	3	0.85	212	29	1.0001011	1500.02
	5	0.83	323	32	1.0001321	1500.06
	7	0.84	401	34	1.0001567	1500.26
	9	0.87	457	37	1.0001893	1500.36

Table 2. Rate and Thermodynamic Constants for Interactions of cAMP and β-CD at 25 °C

Host	Guest	K	k_f	k_b	ΔV	
		M^{-1}	10^8 $M^{-1}s^{-1}$	10^6 s^{-1}	10^{-6} m^3mol^{-1}	
β-CD	cAMP	283 ± 11	4.5 ± 0.2	1.6 ± 0.06	1.22 ± 0.1	this study
β-CD	AMP	89 ± 5	2.1 ± 0.2	2.3 ± 0.1	13.8 ± 0.4	Bae *et al.*[14]

evidently due to the dynamic interactions between β-CD and cAMP. The relaxation from a perturbation of the chemical equilibrium by ultrasonic waves can be described by the following:

$$\beta\text{-CD} + \text{cAMP} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \beta\text{-CD·cAMP} \quad (2)$$

where β-CD is the host, cAMP is the guest molecule, and β-CD·cAMP is the host-guest inclusion complex. k_f and k_b are the forward and backward rate constant, respectively.

The relaxation time, τ, based on chemical relaxation analysis, was calculated from the equation

$$\tau^{-1} = 2\pi f_r = k_b\{(KC_{\beta\text{-}CD} + KC_{cAMP} + 1)^2 - 4K^2C_{\beta\text{-}CD}C_{cAMP}\}^{1/2} \quad (3)$$

where K is the equilibrium constant defined as $K = k_f/k_b$ and $C_{\beta\text{-}CD}$ and C_{cAMP} are the initial concentrations of the host and guest, respectively. The initial concentration of the guest, C_{cAMP}, is the only variable for the relaxation frequency when the concentration of β-CD is kept constant (i.e., $C_{\beta\text{-}CD}$ = 8.7 mM in this study).

Consequently, the two unknown parameters, K and k_b, were estimated using the non-linear least mean square method. The results obtained are listed in Table 2 together with the values of AMP for comparison. Plots of the $2\pi f_r$ versus the concentration term, $\{(KC_{\beta\text{-}CD} + KC_{cAMP} + 1)^2 - 4K^2C_{\beta\text{-}CD}C_{cAMP}\}^{1/2}$, are shown in Figure 3, where the solid line is drawn using the determined K and k_b values. The results clearly demonstrate that the experimental data are correlated well with the calculated line, which supports the 1:1 stoichiometry of the inclusion complex.

The maximum absorption per wavelength, μ_m, which was determined from the ultrasonic absorption measurements, can be related to the standard volume change of the reaction, ΔV, with the aid of the density and sound velocity measurements as follows:

$$\mu_m = \pi\rho\upsilon^2(1/[\beta\text{-CD}] + 1/[\text{cAMP}] + 1/[\beta\text{-CD·cAMP}])^{-1}(\Delta V)^2/2RT$$

where R is the gas constant, and T is the absolute temperature. The equilibrium constant, K, was already determined from the dependence of the concentration on the relaxation frequency. The individual equilibrium concentrations of the reactants could be calculated based on the initial concentrations of the two solutes, β-CD and cAMP. The ΔV values were estimated at each concentration, and the averaged values are given in Table 2.

Discussion

In this study, we demonstrated that the interactions between β-CD and cAMP follow a typical spectrum of single relaxation process that resulted from host-guest interactions.

Cyclic AMP exists in a two-state syn/anti glycosyl conformational equilibrium that prefers an anti conformation over syn conformation in the solid state by a ratio of 7:3.[15] Based on the crystallography studies, both the syn and anti glycosyl conformations of cAMP are found in the active site of PDE with the syn conformation of cAMP occurring in PDE10A2 and the anti conformation of cAMP occurring in PDE4D.[17] Upon cleavage of the phosphodiester bond by PDE, the resulting AMP favors the anti conformation.[18] However, the syn/anti glycosyl conformations of cAMP that resulted from ultrasonic relaxation are only observed when there are high concentrations of cAMP (e.g. greater than 50 mM)[15] and were not observed in our experiments using 10 mM cAMP (see Figure 1). Thus, the ultrasonic relaxations shown in this study can be attributed to perturbation of the host-guest interactions between β-CD and cAMP in aqueous solution.

Inclusion complex studies of β-CD with AMP,[8,14] aspirin[6] and drugs[7] have shown that the forward rate constant, k_f, which is dependent on the diffusion-controlled reaction regardless of guest molecules, is in the order of ~10^8 $M^{-1}s^{-1}$. The forward rate constant, k_f, of β-CD and cAMP obtained in our study was 4.5×10^8 $M^{-1}s^{-1}$, which is also consistent with the values of the diffusion-controlled reactions.[6-8] However, the backward rate

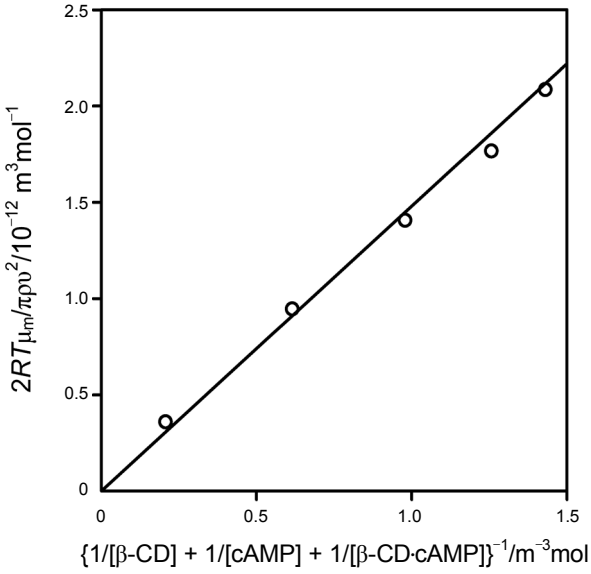

Figure 4. Plots of $2RT\mu_m/\pi\rho\upsilon^2$ *vs*. (1/[β-CD] + 1/[cAMP] + 1/[β-CD·cAMP])$^{-1}$ for aqueous solution of cAMP in the presence of 8.7 mM β-CD at 25 °C.

constant, k_b, is dependent on the structure of the guest molecules. The more hydrophobic the guest molecule, the slower dissociation from the host-guest complex is. If only the hydrophilic character of both the adenine and ribose moieties is considered, the formation of an inclusion complex of cAMP or AMP with the hydrophobic cavity of β-CD cannot be clearly explained.[8] The adenine base has a partial hydrophobic character due to the purine ring. Seno *et al*. suggested that NADH and NADP form stable inclusion complexes with β-CD by incorporating their adenine moiety into the CD cavity.[19] Hao *et al*. reported that the adenine molecule in solution prefers to be included in the more hydrophobic environment inside the β-CD cavity based on enhancement of the fluorescence intensity of adenine in the presence of β-CD.[20] Kondo and Nishikawa reported that adenine and β-CD forms an inclusion complex based on the results of an ultrasonic relaxation study.[8] Other evidence indicating that hydrophobic interactions play important roles comes from the crystal structure of cAMP bound in the binding pocket of PDE. In the crystal structure of PDE4, the binding pocket is lined with conserved hydrophobic and negatively charged residues.[3] The purine base is held tightly in the binding pocket by a hydrophobic clamp formed by a pair of hydrophobic residues,[21,22] and the hydrophobic interactions are believed to provide much of the binding energy in the ligand binding to the binding pocket of PDEs.[22] The catalytic domain of PDE3 has a Michaelis-Menten constant, K_M, of 2 ~ 3 μM for cAMP.[23] Our data indicate that the dissociation constant, K_d, of cAMP binding to β-CD is ~ 3.53 mM, which requires approximately 10^3 more cAMP binding to β-CD to produce the same half-maximal binding. For tighter binding to cAMP, PDE provides ionic interactions and hydrogen bonds in addition to hydrophobic interactions, whereas β-CD provides primarily hydrophobic interactions that lead to weaker interactions. Hydrogen bonds between the hydroxyl groups at the entrance of the β-CD cavity and the polar groups of ribose and phosphate moieties are also partially involved in the inclusion complex formation of adenine nucleotide and β-CD. Thus, AMP, which is more hydrophilic than cAMP due to the hydroxyl group at the 3'-carbon atom, is dissociated faster ($k_b = 2.3 \times 10^6$ s^{-1}) than cAMP is dissociated from β-CD ($k_b = 1.6 \times 10^6$ s^{-1}).

Not only the forward and backward rate constants, but also the standard volume change, ΔV, can be obtained from ultrasonic relaxations. The standard volume change, ΔV, is expressed as $\Delta V = nV_{H_2O} - V_{incl}$, where n is the number of water molecules, V_{H2O} is the molar volume of water and V_{incl} is the molar volume of the guest in the β-CD cavity. Considering that the volume of the β-CD cavity is 2.6×10^{-28} m^3, the entire nucleotide molecule is too large to be included in the cavity.[5] The molar volume of adenine is 89.6×10^{-6} m^{3}mol^{-1}, which corresponds to 14.9×10^{-29} m^3 per molecule.[24] Approximately 1.7 molecules of adenine base can be incorporated into the β-CD cavity. It is known that there are 5 to 7 water molecules in the β-CD cavity.[25] The standard volume change, ΔV, in AMP and cAMP are 13.8×10^{-6} m^{3}mol^{-1} and 1.2×10^{-6} m^{3}mol^{-1}, respectively. Considering that the same adenine base of AMP and cAMP is incorporated into the β-CD cavity, the number of water molecules expelled from the β-CD cavity is 5.7 for AMP and 5.0 for cAMP based on the above equation on the standard volume change. Our results indicate that both AMP and cAMP are effective in replacing the water molecules in the β-CD cavity, while AMP in the anti conformation has a larger chance of collisions with the water molecules in the β-CD cavity than cAMP in the syn conformation, which has restricted freedom of motion.

In conclusion, β-CD and cAMP interacts to form an inclusion complex in aqueous solution. The stability of the complex depends on the hydrophobic interactions between the adenine moiety of cAMP and the β-CD cavity. The syn/anti glycosyl conformations of adenine nucleotides are considered to play an important role in formation of the inclusion complex.

Acknowledgments. We thank Drs. Jinwoo Han and Sei-Heon Jang for valuable discussions. This work was supported by the Daegu University Research Grant, 2008 (to J.-R.B.).

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