Effect of Pressure on Cyclodextrin-catalysed Hydrolysis of Nucleotide 2':3'-Cyclic Monophosphates

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The effect of pressure on the cyclodextrin (CyD)-catalysed regiospecific P–O cleavage of nucleotide 2':3'-cyclic monophosphates (cNMP) was investigated. In all cases hydrolytic reactions in the absence and the presence of CyD were accelerated by increasing pressure, indicating negative activation volume. The apparent regiospecificity seen in the cAMP–β-CyD and cCMP–α-CyD combinations, however, became less prominent at higher pressure. The apparent activation volumes of P–O(3') cleavage of cAMP in the presence of β-CyD (6 cm^3 mol^{-1}) was almost half that in the other cases. The CyD concentration dependence of the ratio of the apparent rate constants of P–O(2') and P–O(3') cleavages was studied and the activation volumes for k_{c2} and k_{c3} for cAMP–β-CyD were calculated as −9.7 and −14.5 cm^3 mol^{-1}, respectively. The apparent decrease in the regiospecificity seen in the reaction cAMP + β-CyD is, therefore, responsible for the positive volume change of the complex formed from cAMP and β-CyD (~5 cm^3 mol^{-1}).

The difference in the activation volumes of the P–O(2') and P–O(3') cleavages of cCMP by α-CyD was also found to be *ca.* 4.4 cm^3 mol^{-1}; in this case the high pressure made the reaction less regiospecific.

Cyclodextrins (CyDs) are some of the most important and widely investigated host–guest host compounds used for the inclusion of various molecules and can undergo a type of 'enzyme-like' (catalytic) reaction.[1] Recently, Komiyama's group found that CyDs could catalyse the alkaline cleavage of nucleotide 2':3'-cyclic monophosphates in a regiospecific manner, similar to that found in the partial reaction of ribonuclease.[2–5]

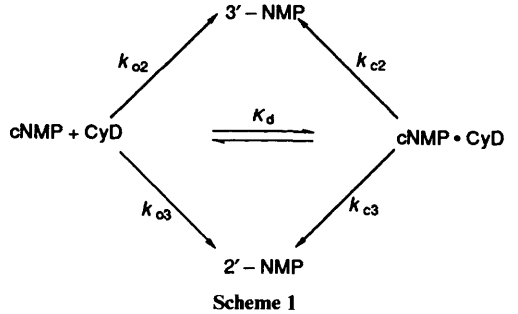
Pressure, an important intensive thermodynamic parameter, affects enzyme reactions in a characteristic manner. We have studied the pressure dependence of the reactions of various proteases and nucleases in order to elucidate their mechanisms.[6–8] In CyD systems, pressure will have a much more straightforward effect on the catalytic reactions, since the effect on the conformation or hydration of the catalyst molecule is less prominent than in enzyme systems; the profiles of the progressing reaction will thus be more clearly available for study. Taniguchi's group first measured the pressure effect on the CyD-catalysed hydrolysis of hydrophobic carboxylate esters[9] and found that both volume changes of the complex formation and activation volumes were dependent on the type (or tightness) of the formed complexes. In the present case of nucleotide phosphate hydrolysis, both the mode of complex formation and the mechanism of the stereospecific cleavage are highly dependent on the size of the base and of the CyD cavity. Therefore, a study of the difference in the pressure response will help in the understanding of reaction mechanisms.

Experimental

Materials.—α-CyD and β-CyD were purchased from Nacalai Tesque Co. Nucleoside 2':3'-cyclic monophosphates (cNMP; cAMP, cGMP, cCMP and cUMP) were obtained from Sigma Chemical Co. Other reagents were commercial (with guarantee) or of chromatographic grade.

Method.—Reaction mixtures usually contained 50 mmol dm^{-3} sodium hydrogen carbonate buffer (pH 11.08 at 0.1 MPa) and 0.1 mmol dm^{-3} cNMP. In reactions under high pressure, the sample solutions were placed in 350 mm^3 polypropylene tips, which were then sealed. They were then pressurized in a high-pressure testing capsule (C7000-19-1, Yamasui Co.). The temperature was kept constant at 20 °C by circulating thermostatted water. Volumetric parameters were calculated by the usual method.[10] The apparent volumetric parameters, however, included the effect of pressure on (i) the equilibrium of the buffer, (ii) the dissociation equilibrium of water and (iii) compression of volume and increase in actual concentration, besides the intrinsic effects on the reactions. All these factors were common to each evaluated activation volume, and from the reference data the sum was roughly estimated to be −3 cm^3 mol^{-1}, the contribution of which was not explicitly corrected in the values shown below.

Sampling of the reaction mixtures at high pressure was done through batch operation. The products were analysed with a reversed-phase HPLC (Shimadzu LC-6AD with Cosmosil 5C-18P). The eluent was 50 mmol dm^{-3} acetate buffer solution containing 5% (v/v) acetonitrile for cAMP and cGMP or 20 mmol dm^{-3} phosphate buffer solution for cCMP and cUMP. To estimate the kinetic parameters, we used equations based on parallel catalytic reactions, as shown in Scheme 1,



Scheme 1

where k_{02} and k_{03} are the rate constants of the spontaneous reactions for P–O(2') and P–O(3') bond cleavages, respectively, and k_{c2} and k_{c3} are the rate constants for the corresponding

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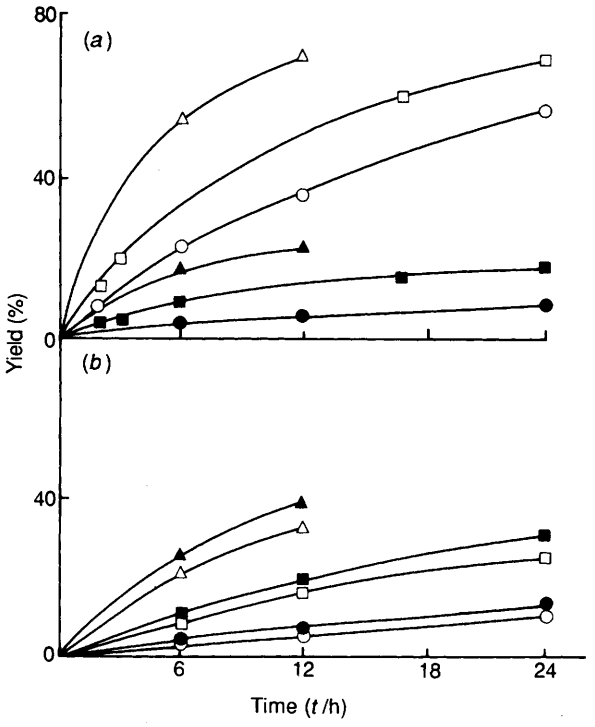

Fig. 1 Time course of the hydrolysis of cAMP in the presence (*a*) and absence (*b*) of β-CyD, at atmospheric and elevated pressure. 50 mol dm^{-3} sodium carbonate (pH 11.08 at 0.1 MPa), 20 °C. [cAMP] = 0.1 mmol dm^{-3}. Open, amount of 2′-AMP produced; closed, amount of 3′-AMP produced. ○, 0.1 MPa; □, 200 MPa; △, 400 MPa.

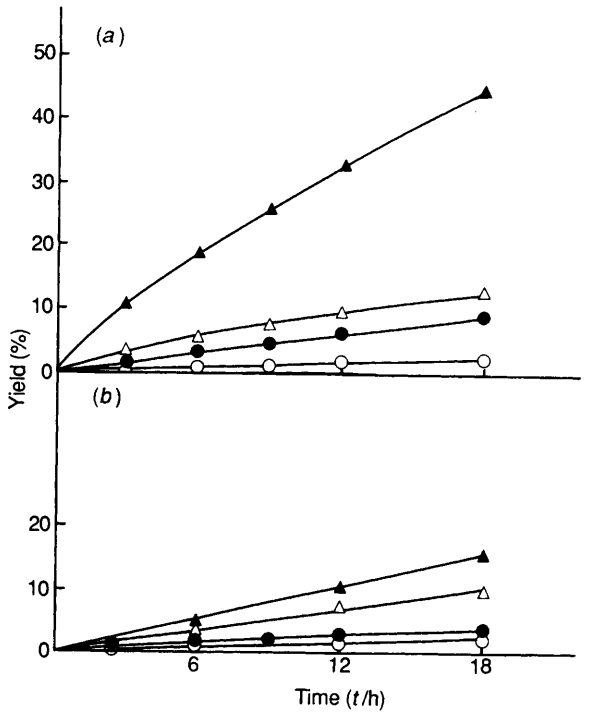

Fig. 2 Time course of the hydrolysis of cCMP in the presence (*a*) and absence (*b*) of α-CyD, at atmospheric and elevated pressure. 50 mmol dm^{-3} sodium carbonate (pH 11.08 at 0.1 MPa), 20 °C. [cCMP] = 0.1 mmol dm^{-3}. Open, amount of 2′-CMP produced; closed, amount of 3′-CMP produced. ○, 0.1 MPa; △, 400 MPa.

CyD-catalysed reactions. K_d is the dissociation constant of the cNMP–CyD complex. The rate (r) of the production of 3′-NMP or 2′-NMP is given by eqns. (1)–(3).

$$r_{(3'NMP)} = d[3'NMP]/dt = k_{02} + k_{c2}([CyD]_0/K_d)\,[cNMP] \quad (1)$$
$$= k_{app2}[cNMP] \quad (1')$$

$$r_{(2'NMP)} = d[2'NMP]/dt = k_{03} + k_{c3}([CyD]_0/K_d)\,[cNMP] \quad (2)$$
$$= k_{app3}[cNMP] \quad (2')$$

and

$$r_{(3'NMP)} + r_{(2'NMP)} = (k_{app2} + k_{app3})\,[cNMP] \quad (3)$$

From $[cNMP]_0 = [cNMP] + [2'NMP] + [3'NMP] + [cNMP{\cdot}CyD]$, eqn. (4) holds, where $k_{app} = (k_{app2} + k_{app3})/(1 + [CyD]_0/K_d)$. Therefore, throughout the reaction, eqns. (5)–(7) hold.

$K_d = [cNMP][CyD]/[cNMP{\cdot}CyD]$, and $[CyD]_0 \gg [cNMP]_0$, $[cNMP] = [cNMP]_0 \exp(-k_{app}t)$ (4)

$$[3'NMP] = (k_{app2}/k_{app})[cNMP]_0\,[1 - \exp(-k_{app}t)] \quad (5)$$

$$[2'NMP] = (k_{app3}/k_{app})[cNMP]_0\,[1 - \exp(-k_{app}t)] \quad (6)$$

and

$$R_{3:2} = [3'NMP]:[2'NMP] = k_{app2}/k_{app3} = k_{02} + k_{c2}([CyD]_0/K_d)/k_{03} + k_{c3}([CyD]_0/K_d \quad (7)$$

From eqn. (4) we can plot the decreasing amount of [cNMP] on a logarithmic scale against time, and obtain k_{app} as the slope. By using the measured ratio of [3′NMP]:[2′NMP], k_{app} can be factorised into $k_{app2'} = k_{app2}/(1 + [CyD]_0/K_d)$ and $k_{app3'} = k_{app3}/(1 + [CyD]_0/K_d)$.

The error limit on the concentration of each component, as determined by HPLC, was found to be less than 0.2%, and that on the r-value obtained for each reaction run was *ca.* 0.6%. The standard deviations of the calculated parameters, such as apparent rate constants, were found to be *ca.* 2%.

Results and Discussion

Fig. 1 shows the time course of the hydrolysis of cAMP in weakly alkaline media, in the presence or absence of β-CyD, at atmospheric or elevated pressure. As was reported, the existence of β-CyD considerably increased the amount of the P–O(3′) cleavage of cAMP (which produced 2′-AMP). In every case increasing pressure considerably accelerated the reaction, indicating that the activation volumes had negative values.

In Fig. 2 similar time courses for the hydrolysis of cCMP are shown. In this case, as was also reported, the existence of α-CyD considerably shifted the product distribution toward the P–O(2′) cleavage side (which produced 3′-AMP). Here again, increasing pressure increased the reaction velocity in all cases, showing activation volumes with negative values and an increase in the negative charge in the transient state, which promoted electrostriction of the water medium.

These reaction curves were analysed, as explained above, and apparent $k_{app3'}$ and $k_{app2'}$ values were evaluated at [CyD] = 0 and 10 mmol dm^{-3}. The numerical data are compiled in Table 1. As seen from the k_{app3}/k_{app2} column of Table 1, the apparent regiospecificity became less prominent at higher pressures.

From these values, the apparent activation volumes ($\Delta V^{\ddagger}_{app}$) of P–O(2′) and P–O(3′) cleavage, in the presence or absence of CyD, were calculated, as listed in Table 2. In most cases, $\Delta V^{\ddagger}$-values were between −11 and −13 $cm^3\,mol^{-1}$. It is notable that the $\Delta V^{\ddagger}$-values of the 3′-cleavage of cAMP in the presence of β-CyD and the $\Delta V^{\ddagger}$-values of the cleavage of cCMP at either side in the presence or the absence of β-CyD, were somewhat smaller (less negative).

Table 1 Apparent rate constants of CyD-catalysed hydrolysis of cNMP

Base	CyD	P/MPa	$k_{app}/10^{-4}$ min^{-1}	$k_{app2}/10^{-4}$ min^{-1}	$k_{app3}/10^{-4}$ min^{-1}	k_{app2}/k_{app3}
A		0.1	1.9	0.90	1.0	0.90
		400	17.0	7.8	9.2	0.85
	α	0.1	2.7	0.96	1.7	0.55
		400	23.0	8.0	15.0	0.54
	β	0.1	7.4	6.4	0.95	6.7
		400	34.5	26.0	8.5	3.1
G		0.1	2.3	1.1	1.2	0.96
		400	19.0	8.0	11.0	0.76
	α	0.1	3.7	1.3	2.4	0.55
		400	20.8	7.8	13.0	0.62
	β	0.1	2.5	1.4	1.1	1.3
		400	20.6	11.0	9.6	1.2
U		0.1	0.80	0.29	0.52	0.52
		400	6.1	2.2	3.9	0.56
	α	0.1	1.1	0.28	0.83	0.34
		400	8.4	2.1	6.2	0.34
	β	0.1	0.79	0.30	0.49	0.61
		400	6.4	2.7	3.7	0.72
C		0.1	0.59	0.23	0.36	0.65
		400	2.7	1.1	1.6	0.69
	α	0.1	1.1	0.23	0.87	0.26
		400	7.8	1.8	6.1	0.29
	β	0.1	0.62	0.26	0.37	0.70
		400	2.7	1.1	1.6	0.72

Table 2 Apparent activation volumes/cm^3 mol^{-1} for hydrolysis of cNMP in the absence or presence of CyD (10 mmol dm^{-3})

Base	Product	Control	+α-CyD	+β-CyD
A	2′-AMP	−12.9	−12.4	−7.5
	3′-AMP	−13.7	−13.1	−12.5
G	2′-GMP	−12.1	−12.6	−11.0
	3′-GMP	−13.6	−13.3	−10.3
U	2′-UMP	−12.5	−12.2	−13.1
	3′-UMP	−12.1	−12.1	−12.2
C	2′-CMP	−9.5	−12.1	−8.9
	3′-CMP	−9.0	−11.5	−8.7

To study the reaction in more detail, the dependence of the ratio of the apparent rate constants (R) on the concentration of CyD was examined at 0.1 and 400 MPa for the cAMP–β-CyD and cCMP–α-CyD combinations (Fig. 3). For the sake of comparison, $k_{app3}:k_{app2}$ ($R_{3:2}$) is shown for the former and $k_{app2}:k_{app3}$ ($R_{2:3}$) is shown for the latter. As given in eqn. (7), the value for R is determined by five independent parameters: k_{02}, k_{03}, k_{c2}, k_{c3} and K_d. Two of these (k_{02} and k_{03}) are obtained from the measurement at [CyD] = 0 (k_{02} = 1.0 s^{-1} at 0.1 MPa and 9.2 s^{-1} at 400 MPa. k_{03} = 0.9 s^{-1} at 0.1 MPa and 7.8 s^{-1} at 400 MPa). Therefore, the concentration profiles were analysed by non-linear regression with the other three parameters. The concentration profiles of cCMP–α-CyD, however, did not give sufficient saturation profiles, although the solubility of α-CyD is somewhat greater than that of β-CyD and the measured concentration range was far wider. Thus, the profiles of this reaction were analysed by two parameters, namely k_{c2}/K_d and k_{c3}/K_d. The values obtained were: for cAMP hydrolysis in the presence of CyD, k_{c2} 0.83 s^{-1}, k_{c3} 16.5 s^{-1} and K_d 0.019 mol dm^{-3} at 0.1 MPa; k_{c2} 4.2 s^{-1}, k_{c3} 184 s^{-1} and K_d 0.085 mol dm^{-3} at 400 MPa. For cCMP hydrolysis in the presence of α-CyD, k_{c2}/K_d 26 mol dm^{-3} s^{-1} and k_{c3}/K_d 0.68 mol dm^{-3} s^{-1} at 0.1 MPa; k_{c2}/K_d 106 mol dm^{-3} s^{-1} and k_{c3}/K_d 5.8 mol dm^{-3} s^{-1} at 400 MPa.

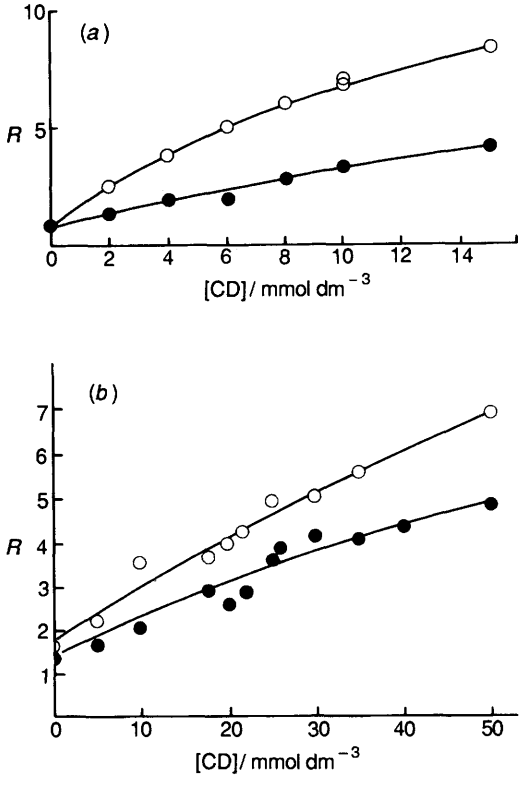

Fig. 3 CyD concentration dependence on R at 0.1 MPa and 400 MPa. 50 mol dm^{-3} sodium carbonate (pH 11.08 at 0.1 MPa), 20 °C. [cNMP] = 0.1 mmol dm^{-3}. (*a*) cAMP hydrolysis in the presence of β-CyD. $R_{3:2}$ is shown. (*b*) cCMP hydrolysis in the presence of α-CyD. $R_{3:2}$ is shown. ○, 0.1 MPa; ●, 400 MPa.

This means that the quotient k_{c2}/k_{c3} was 0.050 at 0.1 MPa and 0.023 at 400 MPa for cAMP + β-CyD, and 37 at 0.1 MPa and 18 at 400 MPa for cCMP + α-CyD. In both cases, increasing pressure reduced the catalytic process at the P–O(2′) cleavage side and, as a result, high pressure forced the cAMP + β-CyD reaction to become more regiospecific.

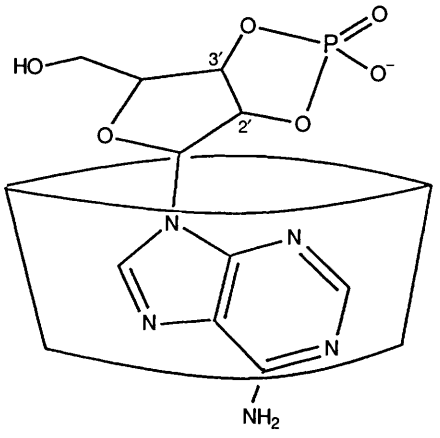

Fig. 4

The activation volumes for reactions k_{c2} and k_{c3} (Scheme 1) for cAMP–β-CyD were evaluated and found to be −9.7 and −14.5 cm^3 mol^{-1}, respectively; the difference in the activation volumes ($\Delta V^{\ddagger}_{k_{c2}} - \Delta V^{\ddagger}_{k_{c3}}$) was *ca.* 4.7 cm^3 mol^{-1}. An apparent decrease in regiospecificity, caused by increasing pressure, was observed for the cAMP + β-CyD reaction [Fig. 3(*b*)]. The smaller apparent $\Delta V^{\ddagger}$-values for the P–O(3′) cleavage of cAMP by β-CyD were, in fact, responsible for the positive volume change of the cAMP–β-CyD complex formation. The rough estimate of this volume change was *ca.* 5 cm^3 mol^{-1}. This value is between the almost zero value estimated for reaction between *p*-nitrophenyl acetate with α- and β-CyD,[9] and the value (*ca.* 10 cm^3 mol^{-1}) given for naphthalene-based fluorescent probes with poly-β-CyD or 2-naphthyl acetate with γ-CyD.[11]

The separated rate parameter showed lower pressure activation for the P–O(2′) cleavage. The transient state of the CyD-catalysed reaction contained the evolution of some negative charges, as in the spontaneous reaction, but the parameter for P–O(2′) (non-specific) cleavage was smaller. Komiyama has proposed a complex of cAMP and β-CyD (Fig. 4).[5] The existence of CyD near the partly generated alkoxide ion at the 2′-position in the transition state may hinder the access of water molecules to the ion and may reduce the number of hydration (electrostricted) water molecules. Recently, addition of an inert salt to the same reaction mixture was found to increase both reaction rate and regiospecificity, if the salt did not inhibit formation of the complex.[12] This phenomenon is related to the difference in the stabilization of the anionic species in the transition state caused by the electric shielding by the salt. An analogy with the effect of pressure and the salt effect was also found in the reaction of metal-containing neutral proteases.[13] In the present case, the decrease in the distance and the strengthening of the hydrogen bonding between the phosphate and the secondary hydroxy groups of the sugar might also contribute to an increase in the negative activation volume in the specific cleavage mode, since the formation of hydrogen bonding generally decreases the molar volume of the system.

The difference in the activation volumes of k_{c2} and k_{c3} for cCMP hydrolysis by α-CyD was 4.4 cm^3 mol^{-1}. High pressure made the reaction (kinetically) less regiospecific, and the regiospecificity is predicted to become inverted at 2000 MPa, when a very high concentration of CyD could be present. In the complexation of cCMP and α-CyD, as proposed by Komiyama

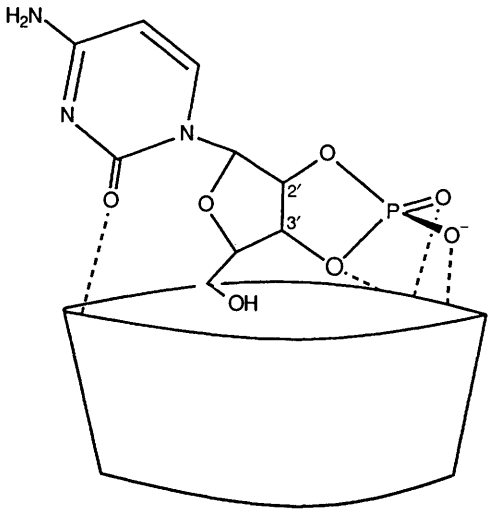

Fig. 5

(Fig. 5),[4] the hydrogen bonding between the cytosine moiety and CyD is important. The pressure might also affect the interactions and solvations of the base moiety of the substrate in the transition state; these influences are accounted for in the evaluated activation volume. The delocalization of the (transient) negative charges, through hydrogen bonding, might be the main reason for the decrease in the degree of hydration of the transition state and the main reason why the $\Delta V^{\ddagger}$-value for specific cleavage becomes more positive.

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