The Effects of Methylated Cyclodextrins on the Transphosphorylation among AMP, ADP and ATP in the Presence of *Escherichia Coli*.

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Transphosphorylation, 2ADP ⇄ AMP+ATP, occurred in the presence of intact *Escherichia coli* (*E. coli* : strain K-12) in neutral phosphate buffer solution, because adenylate kinase existed in both cytoplasma and periplasma of *E. coli*. Cyclodextrin(CD)s accelerated the transphosphorylation. The reaction rate depended on the concentration of CD and the kind of CD. Methylated CDs were more effective than parent CD, especially, *hiptakis*-(2,6-*di*-O-methyl)-β-CD(DMCD) was most effective. Addition of CDs makes neither change of *E. coli* concentration nor the leak of adenylate kinase from periplasma of *E. coli*.

INTRODUCTION

Methylated cyclodextrins have received much attention because of their high solubility in both water and organic solvents and their potency of complex formation[1]. Another attractive ability of methylated CDs has been reported in microbiological fields[2]. In the presence of DMCD or *heptakis*-(2, 3, 6-*tri*-O-methyl)-β-CD(TMCD), an increased *Bordatella pertussis* cell growth was observed; moreover, the enhancement of the pertussis toxin production was observed (100 fold)[3]. While the mechanism of the effects is not yet fully discussed, it is an important example of the utilization of CDs.

We have already reported the interesting transphosphorylation system consisting of methylated CD, adenosides and bivalent ion in neutral solution[4]. In this reaction CD is essential, but the role of CDs was not solved. Watanabe and her coworkers reported that adenylate kinase was located in the periplasma of *Escherichia coli*[5]. In this paper, we report the effect of methylated CDs on the transphosphorylation in the presence of *E. coli* and also suggest a new application of CDs in microbiological fields.

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EXPERIMENTAL

Materials. β-CD was purified by repeated recrystallization from water until no carbohydrate fragments besides β-CD were detected with thin layer chromatography. DMCD and TMCD were prepared and purified according to the previous papers[6]. In order to remove trace organic solvents, the crystalline was refluxed in water, evaporated to dryness and dried *in vacuo*. Reagent grade adenosine-5′-monophosphate (AMP), adenosine-5′-diphosphate (ADP) and adenosine-5′-triphosphate (ATP) were used without further purification. The purity of reagent grade ADP was 95% (1.5% of ATP and 3.5% of AMP were contained as impurity).

Transphosphorylation. The reaction mixture contrained 1/5M phosphate buffer (pH7.0), 3.0mM of $MgCl_2$, 2.85 mM of ADP, intact *E. coli* K-12 cells and CDs. The concentration of CD was varied from 0 to 2.5×10^{-2} M (33 mg/ml). The wet cell was suspended in buffer solution and the suspension include 10^3-10^4 cells/ml of *E. coli* in most experiments. The reagents except three adenosides, were used after sterilization by autoclaving. Adenosides were steriled by UV irradiation. The reaction mixture, in test tubes capped with cotton, was incubated at 40° C in a sterile room. At various intervals, cell growth and ATP production were measured. 5μl of the reaction mixture was sampled and analyzed with a HPLC apparatus equipped with an ionexchange column of DEAE-2SW (0.4*id*x 25cm: TOSOH) and eluted with a 0.1M phosphate buffer of pH 6.5 containing 20% acetonitrile at a flow rate of 0.3 ml/min. Detection was made at 260nm UV absorption at room temperature. ATP formation was confirmed by peaks at a defined retention time. Details of the identification of the ATP and HPLC chromatograms were previously reported[4].

The effect of CD on *E. coli* Leak of cell component caused by CDs was checked by the following way. *E. coli* was suspended in phosphate buffer containing large excess concentration of CDs. After shaking 12h at 40°C, the suspension was ultrafiltrated with ULTRACENT-10 and -30 (TOSOH). Protein in the filtrate was estimated by measuring UV absorption at 260nm and 280nm[7].

RESULTS

Adenylate kinase activity of intact K-12 cells. In the presence of 1.0×10^4 cells/ml of *E. coli*, 2.85×10^{-3}mM of ADP was converted to AMP and ATP, and reached the same equilibrium concentration as other two adenosides. HPLC charts of start of incubation and after 50h are shown in Fig 1a and Fig 1b respectively. Fig 2 shows the time course of ATP formation from

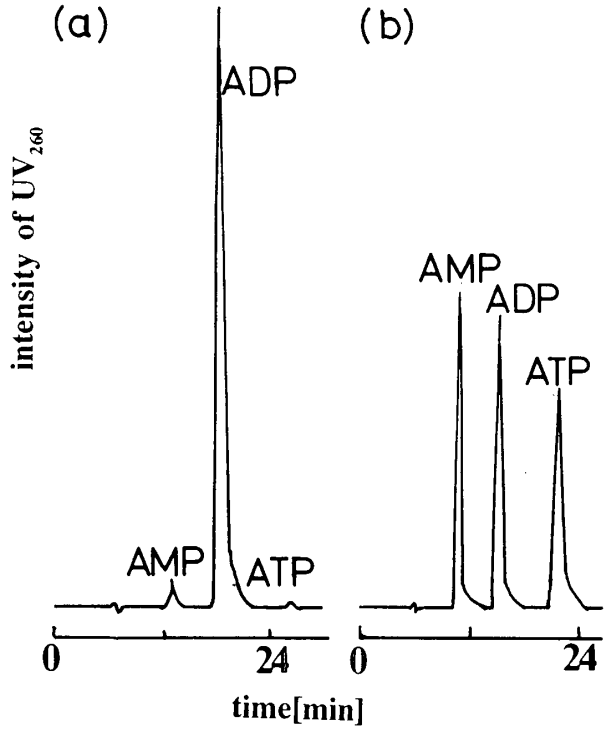

Fig 1. HPLC chart of transphosphorylation in the presence of K-12 cells. The reaction mixture contained 3mM of adenosides, 3mM of $MgCl_2$ and 10^4 cells/ml of K-12 cells in 1/15M phosphate buffer (pH 7.0), column; DEAE-2SW (id: 0.4×25cm), eluent; 20% acetonitrile in 0.2M phosphate buffer (pH 6.5), sampling size; 10μl, detection; UV260nm.

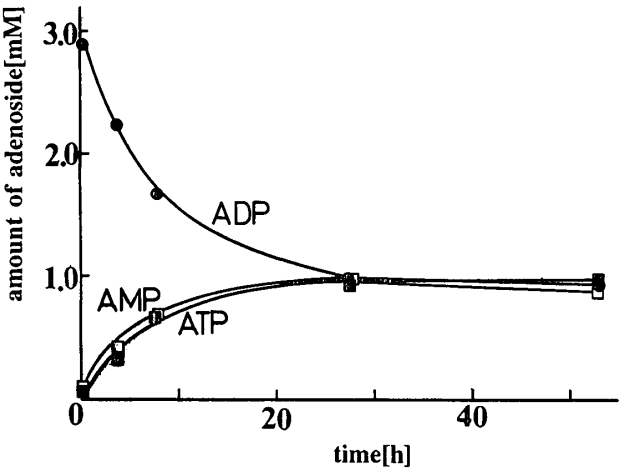

Fig 2. Time course of ATP and AMP formation from ADP in the presence of K-12 cells in 1/15M phosphate buffer. $[AMP]_0=0.10\times10^{-3}$mM, $[ADP]_0=2.85\times10^{-3}$ mM, $[ATP]_0=0.05\times10^{-3}$mM. ●: ADP, □: AMP, ■: ATP.

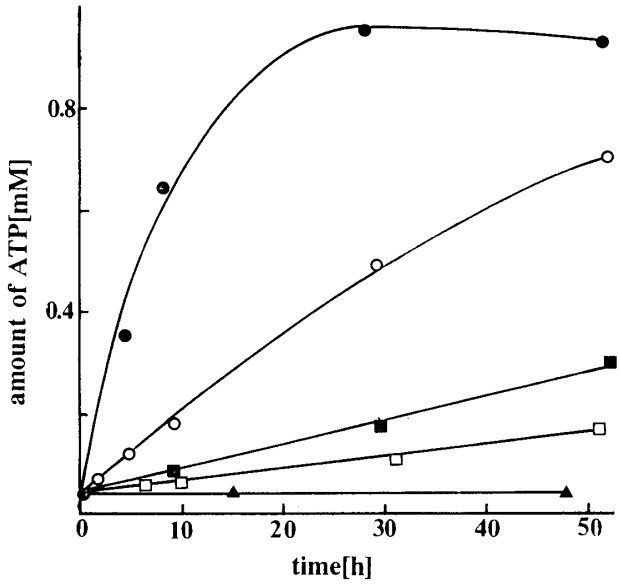

Fig 3. The time course of ATP formation from ADP in the presence of K-12 cells in 1/5M phosphate buffer at 40°C. cell concentration (cells/ml) ; ●: 1.0×10^3, ■: 1.0×10^2, □: 4, :none.

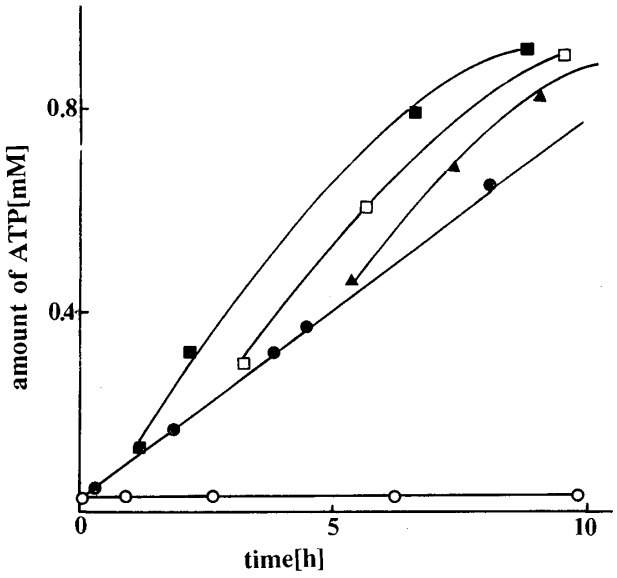

Fig 4. Effect of addition of DMCD on ATP formation in the presence of K-12 cells. DMCD was added to the reaction mixture after 1h (■), 3h (□) and 5h (▲). [K-12] = 4.6×10^4cells/ml, [ADP] = 3mM, [$MgCl_2$] = 3 mM, [DMCD] = 1×10^{-2}M. ●: without DMCD, ○: without both DMCD and K-12 cell.

ADP in the presence of K-12 cells. These results indicated the disappearance of two molecules of ADP and the formation of one molecule of AMP and ATP. Fig 3 shows the time course when the cell concentration of K-12 was varied from 0 to 10^4 cells/ml. In the absence of K-12 cells, ATP and AMP formation was not observed after 200h of incubation. The rate of reaction depended on cell concentration. The cell concentration before and after incubation was almost the same (Fig 6).

The effect of methylated cyclodextrins on the ATP formation in the presence of *E. coli*. Fig 4 shows the effect of 10mM of DMCD on the ATP formation by intact K-12 cells. When DMCD was added at 1,3 and 5h after the start of incubation, ATP formation was accelerated immediately. When DMCD was added after 1h incubation, the concentration of three adenosides reached the approximately equiblium concentration within 10h incubation. ATP formation by K-12 cells depended on the concentration of DMCD (Fig 5). As the concentration of DMCD increased, the ATP formation

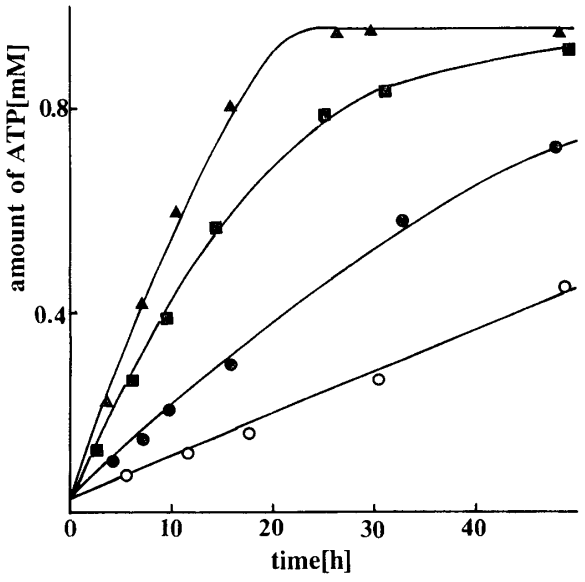

Fig 5. Dependence of ATP formation on DMCD concentration in the presence of K-12 cells. pH 7.0 phosphate buffer, [ADP] = [$MgCl_2$] = 3mM, [DMCD] = 2.5×10^{-2}M(▲), 2.5×10^{-5}M(●), 0(○).

enhanced. In each condition, the concentration of K-12 did not also change (Fig 6).

Fig 7 shows the time course of ATP formation with various CD derivatives. When DMCD was replaced with TMCD or β-CD, the acceler-

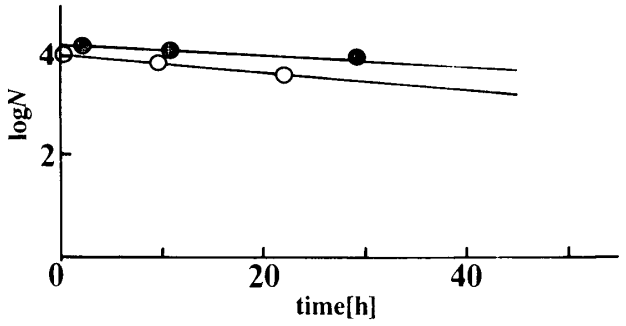

Fig 6. Time course of cell concentration in the absence (◐) or presence (○) of DMCD. N : cell concentration of reaction mixture [cells/ml].

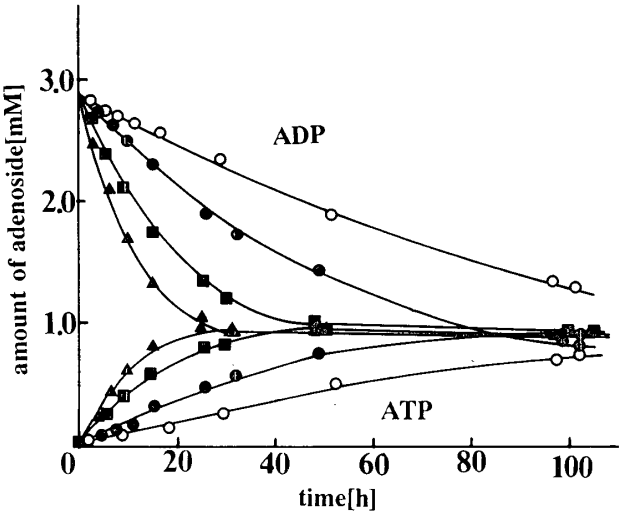

Fig 7. Time course of ATP formation from ADP with K-12 cells in the presence of various CDs. ○ : without CD, ● : β-CD, ■ : TMCD, ▲ : DMCD.

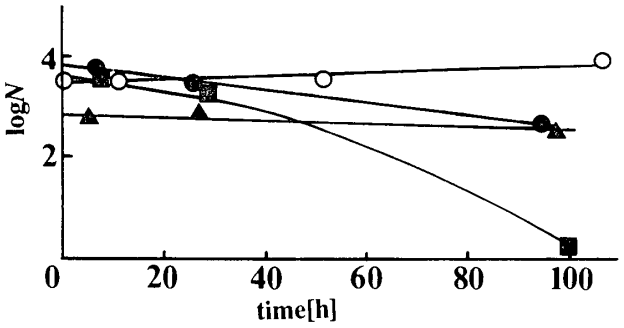

Fig 8. Effect of adding methylated CDs on the cell concentration in 1/15M phosphate buffer with 3mM of $MgCl_2$ within 30h. the concentration of DMCD is 0(○), 2.5×10^{-2}M(▲), 2.5×10^{-3} (■) and 2.5×10^{-5}M(●), and the cell concentration of TMCD is 2.5×10−2M(□).

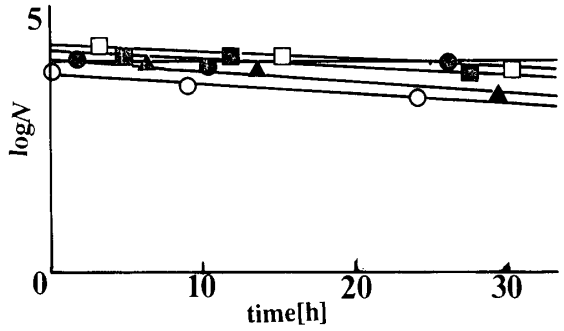

Fig 9. Effect of adding methylated CDs on the cell concentration within 100h. ○ : without CD, ● : β-CD, ▲ : DMCD, ■ : TMCD.

ation of ATP formation was also observed. Without K-12 cells, there were no ATP formation even in excess concentration of CDs. Similar effects of methylated CDs were observed in the reaction mixture in the presence of other strains of *E. coli*.

The effect of methylated cyclodextrins on intact *E. coli*. Fig 8 shows the cell concentration of K-12 in phosphate buffer containing various concentration of CDs. Within 30h, a significant decrease in cell concentration was not observed. But with TMCD, the cell concentration decreased and was almost zero after 100h incubation (Fig 9). Furthermore, there were no differences between the cell growth in M9 medium with and without methylated CD. In the medium consisting of only methylated CD, K-12 cells were not harvested. The estimation of UV spectra indicated that there were no effect on protein leak from *E. coli* caused by DMCD. But TMCD caused the protein leak from the cell.

DISCUSSION

The reaction, 2ADP ⇄ AMP+ATP which is known to be catalyzed by adenylate kinase, occurred in the presence of intact K-12 cells. The reaction had no induction period and the reaction rate depended on the K-12 cell concentrations. It had been previously reported that ATP and AMP were immediately converted into ADP by intact Ad-3 cells of *E. coli* at a

concentration of 5×10^8 cells/ml in the presence of Mg^{2+}, while ADP was also rapidly converted into ATP and AMP under the same conditions. It was also concluded that adenylate kinase in *E. coli* occurred in the periplasma as well as in the cytoplasma[5]. In the K-12 cell of *E. coli*, adenylate kinase also located in the periplasma and the phosphorylation reaction was catalyzed at the same level as in the Ad-3 cell of *E. coli*. When methylated CD was added to the reaction mixture containing K-12 cells, transphosphorylation was immediately accelerated without any change of cell concentration. In the absence of any bacterial strain, no transphosphorylation was observed even with 2.5mM of DMCD addition.

These results indicated that the acceleration was not caused by the enhancement of adenylate kinase concentration owing to the enhancement of *E. coli* concentration or to the release from the cytoplasma by disruption of the cells. This leads to the conclusion that methylated CD interacts with the intact cells at the outer membrane and then promotes the permeation of the reactant; AMP, ADP and ATP.

Uekama and his coworkers had reported that methylated CD has high hemolyzation[6]. These are some evidences that the interaction effect on ATP formation by methylated CD was more remarkable than that of the parent CD. Methylated CD has many methyl groups located on the hem of CD ring, which makes high lipophilicity. Therefore, methylated CD seems to approach the cell membrane more effectively than the parent CD and then induces a decrease in the barrier function of permeation.

In the previous reports, we indicated a new transphosphorylation system which consisted of CD, $MgCl_2$ and adenosides in neutral aqueous solution[4]. In this system, $2ADP \rightleftarrows AMP+ATP$ proceeded at 37°C in 200h. Oxygen bubbling enhanced the reaction rate. However, transphosphorylation was not observed under sterilized conditions. So the role of CD in the system should be the same as that in this *E. coli* medium.

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