Molecular Design of Inhibitors of \textit{in Vitro} ori\textit{C} DNA Replication Based on the Potential to Block the ATP Binding of DnaA Protein*\textsuperscript{a}

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DnaA protein, the initiation factor for chromosomal DNA replication in \textit{Escherichia coli}, is activated by binding to ATP. We earlier reported that 3-acetoxy-2,2'-bi-1H-indol inhibited the ATP binding to DnaA protein (Sasaki, S., Mizushima, T., Hashimoto, T., Maeda, M., and Sekimizu, K. (1994) \textit{Bioorg. Med. Chem. Lett.} 4, 1771–1774). In the present study, derivatives of 3-acetoxy-2,2'-bi-1H-indol with different lengths of aliphatic chains at the 3-O position were synthesized, and their potential to inhibit the ATP binding to DnaA protein was examined. Elongation of the aliphatic chain resulted in inhibition of the ATP binding to DnaA protein at lower concentrations. Among the derivatives, 3-[(11-carboxyundecyl) carbamoylmethoxy]-2,2'-bi-1H-indol (structure 7 (3-CUCM-BI)) exhibited the most potent inhibition with an IC\textsubscript{50} value of 7 \(\mu\)M. The mode of the inhibition was competitive. We further demonstrated that structure 7 (3-CUCM-BI) inhibited DNA replication of the ori\textit{C} plasmid in a system reconstituted from purified proteins. This inhibition was specific for the initiation of DNA replication rather than for the elongation. The inhibition was overcome by preincubation of DnaA protein with ATP. Furthermore, structure 7 (3-CUCM-BI) showed little inhibition on DNA synthesis in the ABC primosome system. We propose that structure 7 (3-CUCM-BI) functions in the \textit{in vitro} ori\textit{C} DNA replication by inhibiting the ATP binding to DnaA protein.

Replication of chromosomal DNA in \textit{Escherichia coli} is regulated at the step of initiation. DnaA protein is the initiator for chromosomal DNA replication (1–3); thus, DnaA protein has been considered to play an important role in regulating DNA replication. DnaA protein has a high affinity for ATP (\(K_a\) = 0.03 \(\mu\)M) and for ADP (\(K_a\) = 0.1 \(\mu\)M) (4). In the ori\textit{C} DNA replication system reconstituted from purified proteins, the ATP binding form of DnaA protein is active in DNA replication, whereas the ADP binding form is inactive (4). These results suggest that the ATP binding to DnaA protein activates the protein; however, the possibilities that the ADP binding to the protein inhibits the activity of DnaA protein in the initiation of ori\textit{C} DNA replication and that the ATP binding to the protein is not essential for the process would need to be excluded. Studies on DnaAcos protein, which loses the affinity for ATP and ADP but is active in the initiation of ori\textit{C} DNA replication in \textit{in vitro}, imply this notion (5, 6). To better understand the requirement of the ATP binding for the initiation of ori\textit{C} DNA replication, development of specific inhibitors for the ATP binding to DnaA protein and examination of their effects on ori\textit{C} DNA replication in \textit{in vitro} are important. The availability of such inhibitors would be good tools to study the biological relevance of the ATP binding to DnaA protein.

We reported that 3-acetoxy-2,2'-bi-1H-indol inhibited the ATP binding of DnaA protein (7). This indol is the first known synthetic organic compound to inhibit the ATP binding of DnaA protein. However, concentration of the drug necessary for inhibition is relatively high, which makes it difficult to examine effects on DNA replication, \textit{in vitro} or \textit{in vivo}. In the present work, we attempted to decrease the IC\textsubscript{50} value of the drug for inhibition of the ATP binding of DnaA protein by introducing hydrophobic residues and carboxyl residue to the 3-acetoxy-2,2'-bi-1H-indol. We obtained evidence that the most potent inhibitory compound, 7 (3-CUCM-BI),\textsuperscript{1} inhibited ori\textit{C} DNA replication in a system reconstituted from purified enzymes through specific inhibition of the ATP binding activity of DnaA protein.

EXPERIMENTAL PROCEDURES

Materials—DnaA protein was purified by the method described elsewhere (8), except that a newly constructed overproducer was used.\textsuperscript{2} Specific activity of the protein was 0.7 \(\times\) 10\textsuperscript{6} units/mg. Purity of the fraction used exceeded 90%, as determined by SDS-polyacrylamide gel electrophoresis.

Synthesis of Inhibitors—All the inhibitors used in this study were synthesized starting with indigo (1) via the key intermediate 2 (3-Ac-BI) (7), as shown in Fig. 1. General—\textit{H}-NMR spectra were taken at 500 or 270 MHz. Chemical shifts are reported in ppm downfield from tetramethylsilane. IR spectra were taken on a JASCO IR Report-100 spectrometer, and mass spectra were obtained with a JEOL JMS DX-300 or D-300 mass spectrometer. Column chromatography was done using silica gel BW290 (150–350 mesh, Fuji Division).

3-Methoxy carbonylmethoxy-2,2'-bi-1H-indol (3 (3-MCM-BI))—A mixture of 2 (3-Ac-BI) (0.992 g, 3.42 mmol) and nBuN\textsubscript{4}OH (3.42 ml, 1M in tetrahydrofurate) in tetrahydrofurate (16 ml) was stirred for 10 min at room temperature under argon atmosphere, followed by the addition of methyl bromoacetate (0.342 ml, 3.42 mmol). After stirring the mixture for an additional 10 min, the solvent was evaporated, and the residue was chromatographed on a silica gel column (acetone:hexane = 3:7) to afford 3 (3-MCM-BI) (0.964 g, 3.01 mmol, 88%) as a white powder. Recrystallization from AcOEt/hexane yielded colorless crystals (0.557 g, 51%). mp 190–192 °C. \textit{H}-NMR (CDCl\textsubscript{3}) \(\delta\) 3.86 (3H, s), 5.09 (2H, s), 6.88 (1H, d, \(J = 0.99, 8.25 \text{ Hz}\)), 7.17–7.00 (4H, m), 7.36 (1H, d), \(J = 0.99, 8.25 \text{ Hz}\).

\textsuperscript{1} The abbreviations used are: (3-CUCM-BI), 3-[N-(11-carboxyundecyl) carbamoylmethoxy]-2,2'-bi-1H-indol; (3-MCM-BI), 3-methoxycarbonylmethoxy-2,2'-bi-1H-indol; (3-Ac-BI), 3-acetoxy-2,2'-bi-1H-indol; (3-KCM-BI), 3-carboxymethoxy-2,2'-bi-1H-indol potassium salt; (3-CXCM-BI), 3-(N-carboxymethyl) carbamoylmethoxy-2,2'-bi-1H-indol; (3CPCM-BI), 3-[N(5-carboxypentyl) carbamoylmethoxy]-2,2'-bi-1H-indol.

\textsuperscript{2} T. Katayama, unpublished data.
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7,532 (1H, dd, J = 0.67, 11.29 Hz), 7,59 (1H, m), 7,68 (1H, dt, J = 0.66, 7,21 Hz), 7,04 (1H, bs, 1H), 11.17 (1H, bs); IR (nujol) cm

0.34 mmol) in N,N-dimethylformamid (5.5 ml) at 5°C, and the mixture was stirred for 3 h at the same temperature and overnight at room temperature. The reaction mixture was filtered, and the filtrate was diluted with AcOEt/benzene (1:1). The organic solvents were washed with brine and then dried over Na2SO4.

Inhibitors of oriC DNA Replication—Inhibitory effects by various compounds on the ATP binding activity of DnaA protein were determined by filter binding assay (4). DnaA protein (1 pmol) was incubated with a compound at 0°C for 15 min, and samples were passed through nitrocellulose filters. The radioactivity remaining on the filter was counted in a liquid scintillation counter.

Assay of Dissociation of the ATP-DnaA Complex—Influence of compounds on the release of ATP from the ATP-DnaA complex was examined as described (4). DnaA protein (1 pmol) and [α-32P]ATP (final concentration, 1 μmol) were incubated at 0°C for 15 min in 40 μl of buffer G, and a synthetic organic compound was added. Incubation was continued at 37°C for various periods. Samples were passed through nitrocellulose filters, and radioactivity remaining on the filter was counted in a liquid scintillation counter.

oriC Replication System In Vivo Reconstituted from Purified Proteins—Reaction mixtures (9) (25 μl) contained 20 mm Tris-HCl (pH 7.4), 0.1 mg/ml bovine serum albumin, 8 μm dihydrothreitol, 0.01% Brij58, 8 μm magnesium acetate, 0.5 μm potassium glutamate, 2 μm ATP, 0.5 μm each of GDP, CTP, and UTP, 200 ng of pBSoriC (600 pmol of nucleotide), 400 ng of SSB, 40 ng of β, 10 ng of HU, 180 ng of GyrA, 360 ng of GyrB, 150 ng of DnaB, 90 ng of DnaC, 10 ng of primase, various amounts of DnaA, 450 ng of DNA polymeraseIII*, and 0.1 μmol of ATP, GTP, dTTP, and α-[32P]TTP (50–150 cpm/μl). Reaction was terminated by chilling on ice and adding 10% trichloroacetic acid. Samples were passed through Whatman GF/cellulose filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter, and the amount of DNA synthesized (pmol nucleotides) was calculated.

RESULTS

Increase in the Inhibitory Activity of 3-Acetoxy-2,2'-bi-IH-indol for the ATP Binding of DnaA Protein by Introduction of Alkyl Chains—We earlier reported that 2 (3-Ac-BI) inhibited the ATP binding of DnaA protein (7). We have now modified this compound to increase the inhibitory activity. Acidic phospholipids, cardiolipin and phosphatidylglycerol, inhibit the ATP binding of DnaA protein (11–14). Fatty acid moieties are essential for inhibition as preincubation of phospholipids with phospholipase A2 diminishes the inhibition (11). We thus assumed that introduction of hydrophobic residues to the bis-indol skeleton would increase the inhibitory activity of the drug to the ATP binding to DnaA protein. We chose the 3-O site of bis-indol as the target for modification. Simple exchange of the acetyl group of 2 (3-Ac-BI) with methoxycarbonylmethyl (3 (3-MCM-BI)) did not increase the inhibitory activity of the drug to the ATP binding to DnaA protein; the IC50 value for the inhibition of the ATP binding of DnaA protein increased about 10 times by this modification (data not shown).

As negative charges of lipid molecules are essential to inhibit the ATP binding to DnaA protein (13, 14), we hydrolyzed the methyl ester residue of 3 (3-MCM-BI) and examined the inhibitory effect on the ATP binding of DnaA protein. The compound with the carboxyl residue of 4 (3-KCM-BI) was 10 times more potent than 3 (3-MCM-BI) (data not shown). Next, we planned to introduce a carboxylalkyl chain onto the structure of 4 (3-KCM-BI). Three amino acid derivatives having alkyl chains of different lengths between the amino and the carboxy residues were condensed with 4 (3-KCM-BI) to produce the inhibitors (5 (3-CPCM-BI), 6 (3-CPCM-BI), and 7 (3-CUCM-BI)). The most potent drug, 7 (3-CUCM-BI), was highly soluble in water. This character is of great advantage for further study on the inhibition of the
drug on DNA replication in vitro.

To study the role of the alkyl chain in the inhibition, we examined the influence of a mixture of 4 (3-KCM-BI) and 12-aminododecanoic acid methyl ester hydrochloride (8) on the ATP binding to DnaA protein. As shown in Fig. 3, the inhibitory activity of the mixture on the ATP binding is less than that of 7 (3-CUCM-BI) and similar to that of 4 (3-KCM-BI). 8 (12-aminododecanoic acid methyl ester hydrochloride) showed little inhibitory effect (Fig. 3). These results suggest that the alkyl chain and bis-indol skeleton cooperate in inhibiting the ATP binding to DnaA protein.

To study the mode of the inhibition of the ATP binding of DnaA protein by 7 (3-CUCM-BI), we examined the titration of ATP on the ATP binding to DnaA protein in the presence and absence of the drug. Double reciprocal plot analysis revealed that the lines in the presence and absence of 7 (3-CUCM-BI) crossed on the Y axis despite that the lines were distinguishable (Fig. 4). The apparent Kd values of the ATP binding to DnaA protein in the presence and absence of the drug were 4 and 0.2 μM, respectively. Therefore, the inhibitory mode of 7 (3-CUCM-BI) on the ATP binding of DnaA protein is competitive.

Stimulation of Dissociation of the ATP-DnaA Complex by Drugs—We examined the influence of organic compounds we synthesized on stability of the ATP-DnaA complex. DnaA protein was preincubated with [α-32P]ATP, and each drug with a concentration 5 times exceeding the IC50 (1 mM, 4 (3-KCM-BI); 280 μM, 5 (3-CMCM-BI); 200 μM, 6 (3-CPMC-BI); 20 μM, 7 (3-CUCM-BI)) was added, followed by incubation at 37 °C. The ATP-DnaA protein complex was stable in the absence of the drug; more than 90% of the complex remained in an intact form after incubation at 37 °C for 5 min under these conditions (data not shown). The exchange rate of ATP bound to DnaA protein is low because even in the presence of excess amounts of non-radiolabeled ATP, the radiolabeled ATP-DnaA complex is stable as described previously (4). All the drugs almost completely inhibited the ATP binding of DnaA protein when added to DnaA protein prior to ATP (data not shown). This result means that the re-association of ATP to free DnaA protein does not occur under these conditions. All the drugs tested increased the dissociation rate of ATP bound to DnaA protein (Fig. 5). The stimulatory activity of drug for dissociation of the ATP-DnaA protein complex was higher when the longer alkyl chains were introduced into the 3-O site of 2 (3-AC-BI) (Fig. 5). Thus, 4 (3-KCM-BI), as well as 2 (3-AC-BI), stimulated little dissociation of the ATP-DnaA complex, whereas 7 (3-CUCM-BI) strongly enhanced the reaction (Fig. 5). The results suggest that the hydrophobic residues introduced contributed to not only inhibition of the ATP binding to DnaA protein but also to stimulation of the dissociation of the ATP-DnaA complex.

We also examined the influence of temperature on the stimulation by 7 (3-CUCM-BI) for the dissociation of the ATP-DnaA protein complex. Stimulation of the dissociation of the ATP-DnaA protein complex by 7 (3-CUCM-BI) required a high temperature; no dissociation was observed at 0 °C, even in the presence of 7 (3-CUCM-BI) (Fig. 6). The result suggests that a higher order structure of DnaA protein is altered at a high temperature, which is probably essential for stimulation of the release of ATP from the complex by the drug. The activation energy for the dissociation of ATP-DnaA complex in the pres-
The presence of 20 μM 7 (3-CUCM-BI) calculated from the Arrhenius plot was 120 kcal/mol, a value somewhat higher than the activation energy for the dissociation of the ATP-DnaA complex in the presence of acidic phospholipid (30 kcal/mol) (7).

Influence of 7 (3-CUCM-BI) on the oriC Plasmid DNA Replication in Vitro in a System Reconstituted from Purified Proteins—Next, we examined the influence of 7 (3-CUCM-BI) on oriC DNA replication in vitro, an event dependent on the function of DnaA protein and requiring the ATP binding to DnaA protein (4). DnaA protein was preincubated with a drug and functioned in the oriC replication system reconstituted from purified proteins. As shown in Fig. 7, the drug inhibited in vitro DNA replication in a dose-dependent manner. The concentration of the drug required for inhibition of oriC DNA replication (IC50 = 60 μM) was higher than that for inhibition of the ATP binding to DnaA protein (IC50 = 7 μM) (Fig. 2).

The reaction in the oriC DNA replication can be separated into two stages: formation of the prepriming complex that requires a high temperature, 38°C, and the following priming and elongation stages that proceed even at a low temperature, 16°C (9). We examined which step was sensitive to 7 (3-CUCM-BI) using the staged reaction. Reaction mixtures depleting primase and DNA polymerase III holoenzyme were incubated at 38°C for 2 min, followed by priming and elongation reactions with radiolabeled deoxyribonucleoside triphosphates at 16°C. When the drug was added after the prepriming complex formation, a much higher concentration of the drug was necessary to inhibit DNA synthesis (Fig. 8), thereby suggesting that the action of the drug is specific for the prepriming stage rather than for the following steps of priming and elongation.

Next, we asked whether the inhibition by 7 (3-CUCM-BI) of oriC DNA replication was caused by inhibitory effects of the drug on the ATP binding to DnaA protein. If such was the case, preincubation of DnaA protein with ATP should diminish the inhibitory action of the drug on oriC DNA replication. On the contrary, if the target of the drug was not specific for the ATP binding of DnaA protein, even after preincubation of DnaA protein with ATP, the drug should have an inhibitory effect on DNA replication. The inhibitory effect of the drug was greatly decreased by preincubation of DnaA protein with ATP (Fig. 9). This means that the drug minimally inhibits oriC DNA replication when DnaA protein is complexed with ATP. The drug may inhibit oriC DNA replication through a specific inhibition.
findings that a number of hydrophobic amino acids cluster near the putative ATP binding domain of DnaA protein (17). Hydrophathy analysis of DnaA protein showed that this region is the most hydrophobic part of the protein (data not shown).

We found that 7 (3-CUCM-BI) potently inhibited the oriC DNA replication reaction reconstituted from purified proteins. The concentration of the drug necessary for inhibition of DNA synthesis was higher when the drug was added after formation of the prepriming complex (Fig. 8). Preincubation of DnaA protein with ATP before the incubation with the drug greatly diminished the inhibitory effect of the drug on oriC DNA replication (Fig. 9). DNA synthesis in the ABC primosome system, which does not require the ATP binding to DnaA protein, was less sensitive to the drug than that in oriC replication system (Fig. 10). These results suggest that 7 (3-CUCM-BI) inhibits oriC DNA replication by inhibiting the ATP binding to DnaA protein. This observation is the first evidence that a specific inhibitor for the ATP binding to DnaA protein inhibits oriC DNA replication, thereby suggesting that the ATP binding to DnaA protein is essential for activity of DnaA protein in the initiation of oriC DNA replication.

The concentration of 7 (3-CUCM-BI) required for inhibition of oriC DNA replication was higher than that for ATP binding to DnaA protein (Figs. 2 and 7). One explanation for this discrepancy is that a certain factor in the oriC DNA replication system decreases the affinity of the drug for DnaA protein. Another is that high concentration of ATP in the system even after DnaA protein had been preincubated with 1 mM ATP at 4 °C for 15 min, followed by incubation with or without 1 mM ATP resulted in increase in affinity of the inhibitor binds to a hydrophobic region near the ATP binding site of DnaA protein resulting in increase in affinity of the drug for DnaA protein. This assumption is supported by

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