Kinetics of ATP Binding to the Origin Recognition Complex of Saccharomyces cerevisiae*

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Origin recognition complex (ORC), a candidate initiator of chromosomal DNA replication in eukaryotes, binds specifically to ATP through two of its subunits (Orc1p and Orc5p). In this study, we investigated the kinetics of ATP binding to ORC by a filter binding assay. The $K_v$ values for the ATP of wild-type ORC and ORC-1A (mutant ORC containing Orc1p with a defective Walker A motif) were less than 10 nM, suggesting that the affinity of Orc5p for ATP is very high. On the other hand, the $K_v$ values for the ATP of ORC-5A (mutant ORC containing Orc5p with a defective Walker A motif) were much higher (about 1.5 $\mu$m), suggesting that the affinity of Orc1p for ATP is relatively low in the absence of origin DNA. ATP dissociated more rapidly from its complex with ORC-5A than from its complex with ORC-1A, suggesting that the ATP-Orc5p complex is more stable than ATP-OrC1p complex. Origin DNA fragments decreased the $K_v$ value of ORC-5A for ATP and stabilized the complex of ATP with ORC-5A. Wild-type ORC, ORC-1A, and ORC-5A required different concentrations of ATP for specific binding to origin DNA. All of these results imply that ATP binding to Orc5p, ATP binding to Orc1p, and origin DNA binding to ORC are co-operatively regulated, which may be important for the initiation of DNA replication.

The initiation of chromosomal DNA replication must be tightly regulated to achieve replication just once per cell cycle; thus, controlling the activity of initiator proteins is important. Adenine nucleotides bound to initiator proteins are involved in this control both in prokaryotes and eukaryotes. In Escherichia coli, DnaA, the initiator of chromosomal DNA replication, has a high affinity for both ATP and ADP (1). The ATP-DnaA complex is active for DNA replication, but the ADP-DnaA complex and nucleotide-free DnaA are inactive (1–3). DnaA has intrinsic ATPase activity, and ATP bound to DnaA can be hydrolyzed to ADP (1). This hydrolysis inactivates the ATP-DnaA complex into the ADP-DnaA complex and suppresses re-replication; in other words, it suppresses over-initiation of DNA replication (4, 5). Acidic phospholipids, such as cardiolipin, interact with the conserved basic amino acid residues of DnaA and stimulate the release of ADP from the ADP-DnaA complex, resulting in re-activation (6–9).

In eukaryotes, origin recognition complex (ORC) is a possible initiator of chromosomal DNA replication (10–16). ORC was originally identified as a six-protein complex (Orc1p to Orc6p) that specifically binds to the Saccharomyces cerevisiae origins of chromosomal DNA replication (16), and its homologues have been found in various eukaryotic species, including humans (17–23). Orc1p has complete consensus sequences of both Walker A and B motifs (presumptive ATP binding sites) (24). Orc5p has a complete consensus sequence of only the Walker A motif (15). ATP binding to Orc1p and Orc5p has been examined by use of mutant ORCs named ORC-1A (containing Orc1p with a defective Walker A motif) and ORC-5A (containing Orc5p with a defective Walker A motif) (25). ORC-5A, but not ORC-1A, required origin DNA (DNA fragments containing an origin of chromosomal DNA replication) for its binding to ATP, suggesting that ATP binding to Orc5p is important for chromosomal DNA replication, but its precise role remains unknown. Furthermore, the kinetics of ATP binding to ORC has not been examined in detail.

A filter binding assay is a good method for examining the kinetics of binding of small molecules to proteins. For example, a filter binding assay of DnaA revealed that it binds to ATP ($K_v$ of 30 nM) and ADP ($K_v$ of 100 nM) and that cardiolipin stimulates the dissociation of ADP from DnaA (1, 27). In this study, we therefore used a filter binding assay to examine the kinetics of ATP binding to ORC. Using wild-type ORC, ORC-1A, and ORC-5A, we determined the $K_v$ values for ATP binding and monitored the dissociation of ATP in the presence or absence of origin DNA. Results show that origin DNA fragments increase the affinity of Orc1p for ATP and stabilized the complex of ATP with Orc1p. It is also suggested that ATP binding to Orc5p increases the affinity of Orc1p for ATP.

EXPERIMENTAL PROCEDURES

Materials—$[^{32}P]$ATP (3000 Ci/mmol), $[^{32}P]$ATP (6000 Ci/mmol), $[^{35}S]$ATP-$\gamma$S (1000 Ci/mmol), and 8-N3-$[^{32}P]$-ATP (azide-modified phosphatase).

* This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by the Asahi Glass Foundation, the Naito Foundation, and the Kato Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ATP Binding to ORC

Results

Establishment of a Filter Binding Assay for ATP Binding to ORC—In this study, we used wild-type ORC, ORC-1A, and ORC-5A, which were purified from over-producing Sf9 cells (Fig. 1). We confirmed that the band between Orc2p and Orc3p is a degradation product of Orc1p by performing immunoblotting analysis using an antibody against Orc1p (data not shown). The fact that the Orc1p of some part of our ORC preparation is degraded may not significantly affect our results in this paper, because we could basically repeat the results in the previous report even though the degradation product of Orc1p was not seen in the previous paper (25).

In the previous paper, ATP binding to ORC was measured in buffer H (50 mM HEPES-KOH (pH 7.5), 0.15 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM MgOAc, 0.02% Nonidet P-40, and 10% glycerol) by use of a gel filtration assay (25). However, buffer H was not suitable for a filter binding assay; in the presence or absence of origin DNA (wild-type ARS1), ATP binding to ORC was more efficiently detected by a filter binding assay in buffer T (25 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 70 mM KCl, 5 mM dithiothreitol, and 5% (v/v) glycerol) than in buffer H (data not shown). Using unlabeled ATP, ADP, CTP, GTP, and TTP, we performed competition assays for ATP binding to ORC and confirmed that results from the filter binding assay in buffer T were similar to those previously obtained from the gel filtration assay in buffer H (25) (data not shown). In the following experiments we therefore measured ATP binding to ORC in buffer T by using a filter binding assay.

Affinity of ORC for ATP in the Presence or Absence of Origin DNA—ATP bound to wild-type ORC and ORC-1A very efficiently, even at low concentrations of ATP (Fig. 2). On the other hand, less ATP bound to ORC-5A even in the presence of higher concentrations of ATP (Figs. 2 and 3). These results suggest that the affinity of ORC-5A for ATP is lower than the affinities of wild-type ORC and ORC-1A. To confirm this, we determined the $K_d$ value of each ORC (Fig. 3 and Table I). The $K_d$ values of wild-type ORC and ORC-1A for ATP were <10 nM, whereas that of ORC-5A was >1 μM in the absence of DNA (Table I).

It was reported previously that origin DNA stimulated ATP binding to wild-type ORC and ORC-5A (25). We examined the effect of origin DNA (wild-type ARS1) on the affinity of ORC for ATP and the amount of ATP bound to ORC by the filter binding assay. ARS1 contains four elements important for its origin function (A, B1, B2, and B3) (29), among which A and B1 are ORC binding sites (16, 26). Thus, we also examined the effect of mutant ARS1, which has mutations in the A and B1 elements (16, 26). As shown in Fig. 3 and Table I, wild-type ARS1 DNA fragments did not affect the $K_d$ value of wild-type ORC for ATP, but they increased the amount of ATP bound to wild-type ORC, which is consistent with a previous report (25). Mutant ARS1
DNA fragments also increased the amount of ATP bound to wild-type ORC, but less so than wild-type ARS1 DNA fragments did (Fig. 3 and Table I). In ORC-1A, wild-type ARS1 DNA fragments affected neither the $K_d$ value for ATP nor the amount of ATP bound to ORC-1A. On the other hand, in ORC-5A, wild-type ARS1 DNA fragments dramatically decreased the $K_d$ value for ATP and also increased the amount of ATP bound to ORC-5A. In ORC-5A, mutant ARS1 DNA fragments affected the ATP binding in a similar way to wild-type fragments, but to a lesser extent. All of these results suggest that origin DNA stimulates ATP binding to Orc1p by increasing the amount of ATP bound to Orc1p and decreasing $K_d$.

ORC has intrinsic ATPase activity that seems to reside on subunit Orc1p, because the activity is seen in ORC-5A but not in ORC-1A. Double-stranded origin DNA fragments can inhibit this activity (25). To test whether ATPase activity affected the results shown in Figs. 2 and 3, we examined ORC binding to ATP-$\gamma$-S, an analogue of ATP that is more difficult to hydrolyze. Most results with ATP (Fig. 4A) were repeated with ATP-$\gamma$-S (Fig. 4B). The most striking difference was that, in the absence of origin DNA, less ATP-$\gamma$-S bound to ORC-5A (Fig. 4B). This is because the affinity of ORC-5A for ATP-$\gamma$-S was weaker than its affinity for ATP ($K_d > 20$ $\mu$M versus $K_d = 1.5$ $\mu$M) in the absence of DNA, and, thus, ORC-5A may bind ATP-$\gamma$-S only when its affinity is increased by origin DNA. The results in Fig. 4A were also repeated with [γ-32P]ATP (data not shown). Therefore, it seems that ATPase activity of ORC did not affect results shown in Figs. 2 and 3 and that those results mainly reflect the binding between complex and ATP unaffected by hydrolysis of the bound nucleotide. We also tried to detect the ATP hydrolysis by a filter binding assay. However, because the complex of ORC with ADP was very unstable, we could not detect the ATP hydrolysis on ORC by a filter binding assay.

Identification of ORC Subunits That Bind ATP by UV Cross-linking—To identify which ORC subunits bind ATP under the conditions of Fig. 3 (in buffer T), we performed UV cross-linking analysis by use of radiolabeled 8-N3-ATP. As shown in Fig. 5, results were basically similar to those reported previously with buffer H (25). In wild-type ORC, Orc5p was labeled in the absence of origin DNA, and Orc1p, Orc4p, and Orc5p were labeled in the presence of origin DNA. For ORC-1A, Orc5p was labeled in both absence and presence of origin DNA. For ORC-5A, Orc1p and Orc4p were labeled in the presence of origin DNA, but not in the absence of origin DNA. For ORC-5A, Orc1p and Orc4p were labeled in the presence of origin DNA, but not in the absence of origin DNA. For wild-type ORC, the relative efficiency of cross-linking for each subunit is different from a previous report (25). Previously, in the presence of origin DNA, Orc1p and Orc4p were more efficiently

\[ H. \text{ Takenada, M. Makise, W. Kuwae, N. Takahashi, T. Tsuchiya, and T. Mizushima, unpublished data.}\]
labeled than Orc5p; but under our conditions the opposite was true (Fig. 5). This difference is due to the difference in the buffer (buffer T in this study and buffer H in the previous study). We found that the cross-linking of Orc5p or Orc1p and Orc4p was more or less efficient, respectively, in buffer T than in buffer H (data not shown). Furthermore, we found that, for wild-type ORC and ORC-1A but not for ORC-5A, Orc3p was labeled in an independent manner of origin DNA (Fig. 5). It seems that the labeling of Orc3p required ATP binding to Orc5p. We confirmed that the addition of 100-fold higher concentrations of unlabeled ATP diminished the labeling of all ORC subunits (data not shown).

The results in Fig. 5 are not completely consistent with the results in Fig. 3. For example, ATP binding to ORC-5A in the absence of origin DNA (Fig. 3) was not clearly observed in Fig. 5 (however, longer exposure of autoradiography made the band of Orc1p visible (data not shown)). This may be because a more stable complex is required for detection by UV cross-linking analysis than for detection by filter binding assay or because the affinity of ORC-5A for 8-N3-ATP is lower than for ATP.

**Dissociation of ATP from ORC-ATP Complex**—As the filter binding assay requires only a few seconds, it enabled us to measure the rate of ATP dissociation from ATP-ORC complexes. After formation of a complex of each ORC with radio-labeled ATP, the complex was incubated in the presence of excess unlabeled ATP, and the release of radiolabel was monitored. As shown in Fig. 6, ATP dissociated more rapidly from ORC-5A than from ORC-1A: 5 min after the addition of unlabeled ATP, <5% of ATP remained on ORC-5A, but >80% of ATP remained on ORC-1A. The \( k_{\text{app}} \) values for ORC-1A and ORC-5A in the absence of origin DNA were 5.3 \( \times \) 10\(^{-4}\) and 9.6 \( \times \) 10\(^{-3}\) (s\(^{-1}\)), respectively. Therefore, the Orc5p-ATP complex in ORC-1A seems to be more stable than the Orc1p-ATP complex of ORC-5A. The dissociation of ATP from wild-type ORC seemed biphasic, as about half of ATP dissociated very rapidly, but the rest dissociated very slowly. It may be that in the wild-type ORC in buffer T, the Orc5p-ATP complex is more stable than the Orc1p-ATP complex.

We also examined the effect of origin DNA on the dissociation of ATP from the complex of each ORC with ATP. As shown in Fig. 7, wild-type ARS1 DNA fragments stabilized the ATP-ORC-5A complex. The \( k_{\text{app}} \) values for ORC-5A in the presence of origin DNA and mutant DNA fragments were 2.7 \( \times \) 10\(^{-3}\) and...
Neither wild-type ARS1 DNA fragments nor mutant fragments affected the stability of the complexes of wild-type ORC with ATP or ORC-1A with ATP (Fig. 7). Therefore, it seems that origin DNA specifically affects the stability of the Orc1p-ATP complex.

**Specificity of ORC Binding to Origin DNA in the Presence of Various Concentrations of ATP**—In the previous report, by using DNase I footprinting analysis it was shown that wild-type ORC and ORC-5A could bind to origin DNA in a sequence-specific manner in the presence of either low (100 nM) or high (10 mM) concentrations of ATP; but ORC-1A could bind to origin DNA in a sequence-specific manner only in the presence of a high concentration of ATP (25). Here, we examined the specificity of the binding of each ORC to origin DNA by use of a filter binding assay, which is more quantitative. We examined the extent of binding of each ORC to radiolabeled wild-type ARS1 or mutant ARS1 (A-B1') DNA fragments in the presence of various concentrations of nonspecific competitor DNA and ATP (5 mM or 5 μM) (Fig. 8). In the presence of either 5 mM or 5 μM ATP, wild-type ORC and ORC-5A bound more wild-type ARS1 DNA fragments than mutant ARS1 DNA fragments (Fig. 8), suggesting that wild-type ORC and ORC-5A can bind to origin DNA in a sequence-specific manner under these conditions. We also compared the specificity of the binding of wild-type ORC and ORC-5A to origin DNA in the presence of various concentrations of ATP. In the presence of 500 nM ATP, a little more of the wild-type ARS1 DNA fragments bound to wild-type ORC than to ORC-5A (data not shown). On the other hand, ORC-1A bound to origin DNA in a sequence-specific manner in the presence of a high concentration of ATP (5 mM) but not a low concentration of ATP (5 μM) (Fig. 8), suggesting that ATP binding to Orc1p is important for its specific binding to origin DNA.
ATP Binding to ORC

DISCUSSION

In this study, we established a filter binding assay for ATP binding to ORC and studied the kinetics of ATP binding to ORC using wild-type and mutant ORCs (ORC-1A and ORC-5A). UV cross-linking analysis with radiolabeled 8-N3-ATP showed that both Orc1p and Orc5p were labeled in wild-type ORC, and either Orc1p or Orc5p were labeled in ORC-5A or ORC-1A, respectively. Labeling of Orc4p depended on ATP binding to Orc1p, as reported previously (25). Furthermore, labeling of Orc3p depended on ATP binding to Orc5p. The labeling of Orc4p was probably not because Orc4p itself binds ATP but because Orc4p is very near the ATP binding site of Orc1p (25). We consider that a similar mechanism can explain the labeling of Orc3p, which has no ATP binding consensus sequences. Therefore, in wild-type ORC, ATP binds mainly to both Orc1p and Orc5p, and in ORC-1A or ORC-5A it binds mainly to either Orc5p or Orc1p, respectively. In the absence of origin DNA, the Kd value of ORC-5A for ATP was much higher than that of ORC-1A, suggesting that the affinity of Orc1p for ATP is much lower than that of Orc5p in the absence of origin DNA. ATP dissociated more rapidly from its complex with ORC-5A than from its complex with ORC-1A, suggesting that the ATP-ORC5p complex is more stable than the ATP-ORC1p complex.

As for the role of ATP binding to Orc5p in chromosomal DNA replication, little information has been reported. We consider that the results in this paper suggest that ATP binding to Orc5p increases the affinity of Orc1p for ATP based on following discussion. In the absence of origin DNA, a greater amount of ATP bound to wild-type ORC than to ORC-1A in the presence of low ATP concentrations (Fig. 2). Furthermore, the amount of ATP bound to wild-type ORC did not increase when the ATP concentration was increased up to 10 μM (data not shown), suggesting that ATP binds to Orc1p of wild-type ORC even in the presence of low ATP concentrations under the conditions of absence of origin DNA. It is clear that ATP binds to Orc5p of wild-type ORC in the presence of origin DNA based on results from UV cross-linking analysis. On the other hand, compared with wild-type ORC, the Kd value of ORC-5A for ATP was higher not only in the absence but also in the presence of origin DNA (Table I), and Scatchard plot analysis of wild-type ORC showed the single low Kd value. Therefore, it seems that the affinity of Orc1p for ATP in wild-type ORC is higher than that in ORC-5A; in other words, ATP binding to Orc5p increases the affinity of ATP for Orc1p. This finding is an important clue to understanding the role of ATP binding to Orc5p in chromosomal DNA replication. In the temperature-sensitive orc5-A mutant (expressing ORC-5A instead of wild-type ORC), the complex became sensitive to protein degradation at non-permissive temperatures. One possible explanation is that in ORC-5A, the lack of ATP binding to Orc5p inhibits the ATP binding to Orc1p, thus inhibiting the ORC binding to origin DNA and leaving the entire complex sensitive to protein degradation. Thus, ATP binding to Orc5p may regulate both ORC stability and ORC binding to origin DNA through the regulation of ATP binding to Orc1p.

In the gel filtration assay, origin DNA stimulated ATP binding to ORC-5A and wild-type ORC (25). We separated the effect into the Kd value and the amount of ATP bound to ORC. For ORC-5A, origin DNA decreases the Kd value and increases the amount of ATP bound to ORC (Table I), suggesting that it decreases the Kd value of Orc1p for ATP and increases the amount of ATP bound to Orc1p. Origin DNA stabilized the highly unstable ATP-ORC-5A complex (Fig. 7), presumably by stabilizing Orc1p-ATP binding. It seems that origin DNA stimulates ATP binding to ORC-5A by stabilizing the Orc1p-ATP complex.

Acknowledgments—We thank Dr. Bruce Stillman (Cold Spring Harbor Laboratory) for providing antibodies against ORC and Dr. Stephen P. Bell (Massachusetts Institute of Technology) for providing recombinant baculoviruses (ORC-1A and ORC-5A).

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N. Takahashi, Y. Yamaguchi, F. Yamaira, M. Makise, H. Takenada, W. Kuwae, T. Tsuichiya, and T. Mizushima, submitted for publication.
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*J. Biol. Chem. 2003, 278:46440-46445.*

doi: 10.1074/jbc.M307392200 originally published online September 8, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307392200

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