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Phospholipids Promote Dissociation of ADP from the *Mycobacterium avium* DnaA Protein[1]

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The biochemical aspects of the initiation of DNA replication in *Mycobacterium avium* are unknown. As a first step towards understanding this process, *M. avium* DnaA protein, the counterpart of *Escherichia coli* replication initiator protein, was overproduced in *E. coli* with an N-terminal histidine tag and purified to homogeneity on a nickel affinity column. The recombinant DnaA protein bound both ATP and ADP with high affinity and showed a weak ATPase activity. ADP, following the hydrolysis of ATP, remained bound to the protein strongly and the exchange of ATP for bound ADP was found to be weak. Acidic phopsholipids such as phosphatidylinositol, phosphatidylglycerol, and cardiolipin, promoted the dissociation of ADP from the DnaA protein, whereas the neutral phospholipid, phosphatidylethanolamine, did not. The phospholipid promoted dissociation of ADP from DnaA protein was stimulated in the presence of the *M. avium* origin of replication. We suggest that the initiation of DNA replication in *M. avium* involves an interplay among DnaA, adenine nucleotides and phospholipids.

Key words: DnaA, DnaA–adenine nucleotide interaction, *Mycobacterium avium*, *ori*C, phospholipids.

Mycobacterium avium-intracellulare (MAC) complex group organisms are the most common cause of mycobacterial lung diseases other than tuberculosis, and are some of the leading causes of morbidity and mortality in AIDS patients (*1*). Together they represent the common bacterial opportunistic infectious agents in AIDS patients that account for approximately 35 to 40% of cases. *M. avium*, a Gram-positive, acid-fast, aerobic bacterium, is a slow grower with a typical generation time of 10 to 12 h (*2, 3*). The genus Mycobacterium also includes other notable pathogens such as *M. tuberculosis*, *M. bovis*, and *M. leprae*, and non-pathogens such as *M. smegmatis* and *M. fortitum*. The doubling times of these organisms range from 2 to 3 h (*M. smegmatis, M. fortuitum*) to 22–24 h (*M. tuberculosis, M. bovis*) to 185 h (*M. leprae*). The genetic and biochemical factors responsible for the differences in the growth rates of various mycobacteria are largely unknown.

The replication initiation process in *Escherichia coli* has been a paradigm for related processes in other organisms (*4–7*). Detailed biochemical studies with the *E. coli* replication initiator protein, DnaA, revealed that the initiation of DNA replication occurs when the DnaA protein binds to DnaA-boxes, the nine nucleotide long DnaA protein recognition sequences located in the origin of replication or also called the *ori*C, and triggers the formation of an initiation complex. The DnaA protein mediated *ori*C replication process is ATP-dependent (*8, 9*). Cooperative binding of DnaA-ATP complexes to nearby AT-rich repeats results in unwinding of *ori*C (*10*). This unwound DNA provides the entry site for the DnaB helicase–DnaC complex and other proteins that ultimately lead to the establishment of bidirectional replication forks (*7, 11*). The *E. coli* DnaA protein has a high affinity for both ATP and ADP (*8*). ATP bound to DnaA protein is hydrolyzed to ADP by its intrinsic ATPase activity, and ADP is strongly retained by the DnaA protein (*12, 13*). Furthermore, it has been shown that the ADP-DnaA complexes are not competent for replication initiation (*8, 14*). Recent studies also revealed that loading the β subunit of the DNA polymerase III at the end of the initiation process stimulates the intrinsic ATPase activity of DnaA. This results in the accumulation of DnaA-ADP complexes and inactivation of DnaA protein (*13, 15*). Acidic phospholipids promote dissociation of strongly bound ADP from the DnaA and help rejuvenate moribund DnaA to an active species competent for replication reinitiation (*16, 17*). DnaA protein has been shown to be associated with membrane fractions (*18*), and acidic phosholipids are believed to be involved in the regulation of DNA replication (*19*). DnaA proteins from a few other organisms, for example, *Bacillus subtilis* (*20*), *Streptomyces coelicolor* (*21*), and *Thermus aquaticus* (*22*), have been purified, and the interactions of DnaA with respect to *ori*C and DnaA-boxes have been investigated.

In marked contrast to the situation in *E. coli*, the genetic and biochemical aspects of replication initiation in *M. avium* are largely unknown. Thus far, only the key elements involved in the replication initiation process, namely *ori*C and DnaA, have been identified (*23*). The *dnaA-dnaN*

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Abbreviations: *ori*C, origin of replication; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

intergenic region of *M. avium* has been shown to function as *oriC* (*23*). The actual process by which the initiation of replication in *M. avium* occurs is however, unknown. As a first step towards understanding the biochemical aspects of DNA replication in *M. avium*, we purified the recombinant DnaA protein and investigated its interactions with adenine nucleotides. Our results indicate that the recombinant *M. avium* DnaA protein interacts with both ATP and ADP with high affinity, exhibits ATPase activity, but retains ADP following the hydrolysis of ATP. We also show that the exchange of ATP for bound ADP is feeble and that acidic phospholipids promote the dissociation of ADP from the DnaA. We suggest that an interplay among DnaA, adenine nucleotides and acidic phospholipids leads to replication initiation in *M. avium*.

MATERIALS AND METHODS

Reagents—Radioactive materials [α-^{32}P]ATP (3,000 Ci/mmol) and [^{14}C]ADP (54.7 Ci/mmol) were purchased from NEN Radiochemicals. All other chemicals, unless otherwise mentioned, were obtained from Sigma Chemicals.

DNA—pZero plasmid DNA was from Invitrogen Corporation. *M. avium oriC* plasmid containing *dnaA-dnaN* intergenic region (570 bp) has been described previously (*23*).

Phospholipids—Cardiolipin (CL, from bovine heart), phosphatidylglycerol (PG, from egg yolk), phosphatidylethanolamine (PE, synthetic), and phosphatidylinositol (soybean) were purchased from Sigma. Phospholipids (10 mg in methanol–chloroform) were dried under a flow of nitrogen and suspended in 1 ml of distilled water by sonication. Phosphatidylethanolamine was suspended in 0.1% Triton X-100.

Construction of Plasmid Overexpressing M. avium dnaA—*M. avium* DnaA protein was overexpressed under the conditions described by Madiraju *et al.* (*23*).

Overproduction and Purification of M. avium DnaA—To characterize the biochemical properties of the DnaA protein, we overproduced it as a His-tagged protein. *E. coli* BL21 (DE3) carrying the *dnaA* overexpression plasmid was grown at 37°C to an A_{600} of 0.8. DnaA protein production was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM for 3 h, and cultures were harvested by centrifugation. The resultant cell pellet was resuspended in the starting buffer [100 mM NaH_2PO_4, 10 mM Tris-HCl (pH 8.0), 6 M urea, and 10 mM imidazole], and disrupted by sonication. After centrifugation, the clear supernatant was loaded on a Ni^{2+}-nitrilotriacetic acid–agarose column (Qiagen) pre-equilibrated with the starting buffer. After washing with the same buffer, the DnaA protein was eluted with imidazole, electrophoretically separated by SDS-PAGE (*24*), and visualized by staining with Coomassie Brilliant Blue R-250 (*23*). Fractions containing the eluted DnaA protein were dialyzed sequentially in 25 mM Tris-acetate (pH 7.5), 250 mM NaCl, 0.1 mM EDTA, 10 mM magnesium acetate, 10 mM 2-mercaptoethanol, and 20% glycerol containing 4, 2, 1, 0.5, and 0 M urea. After brief centrifugation, the supernatant with soluble recombinant DnaA protein was collected and stored at −70°C until use.

ATP (ADP)-Binding Assay—Nucleotide binding experiments were carried out as described by Sekimizu *et al.* (*8*). Binding of ATP to DnaA was carried out in 100 μl of E-buffer [50 mM Tris-acetate (pH 8.25), 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM ammonium sulfate, 20% glycerol, 0.005% Tween20] containing various concentrations of [α-^{32}P]ATP and DnaA protein (1 pmol). Binding of ADP to DnaA was carried out in 700 μl of E-buffer containing various concentrations of [^{14}C]ADP and DnaA protein (7 pmol). After incubation at 37°C for 15 min, the reaction mixture was filtered through a nitrocellulose membrane (Millipore HA 0.45 μM, 24-mm diameter) presoaked in E-buffer. The filter was washed with 10 ml of ice-cold E-buffer, dried, and the amount of radioactivity retained on the membrane was measured in a liquid scintillation counter.

Influence of Phospholipids on the Release of ADP from the ADP-DnaA Complex—The protocols of Sekimizu *et al.* were used to analyze the dissociation of bound nucleotide (*25*). DnaA (10 pmol) was incubated with [^{14}C]ADP (732 pmol) E-buffer (100 μl) for 15 min to prepare the DnaA-ADP complex. Different concentrations of phospholipid vesicles were added to the reaction mixtures and the mixtures were transferred to a 37°C waterbath for 15 min. At the end of the incubation, samples were collected on a nitrocellulose membrane, washed with E-buffer, and the amount of the radioactivity remaining on the filter was counted as described above.

ATPase Activity—Reactions were performed in 40 μl of E-buffer containing [α-^{32}P]ATP (100 pmol) and DnaA protein (40 pmol) in the absence or presence of *M. avium oriC* (0.2 μg). Samples were incubated at 0°C for 15 min prior to transfer to 37°C. At the indicated time intervals, samples were collected on membranes and the bound nucleotides were extracted with 50 μl of 1 M HCOOH. An aliquot (0.5 μl) of the extracted samples was spotted on a polyethyleneimine (PEI)-cellulose (Macherey-Nagel) plate, chromatographed in 1 M HCOOH–0.5 M LiCl as described (*8*), and the plates were dried. Spots were visualized in a Molecular Imager (Bio-Rad), and quantitated using the Quantity One program (Bio-Rad).

RESULTS AND DISCUSSION

Purification of M. avium DnaA Protein—To begin investigating the biochemical properties of the *M. avium* DnaA protein, we cloned and expressed the *M. avium dnaA* gene as a his-DnaA fusion protein in *E. coli* under T7 transcription and translation signals. Initial analyses of the cellular lysates revealed that much of the overproduced recombinant protein was in the inclusion bodies (data not shown). Consequently, purification under urea-denaturing conditions was sought and the recombinant DnaA protein was purified to near homogeneity on two successive columns of Ni^{2+}-nitrilotriacetic acid-agarose. The purified protein was gradually refolded by slow dialysis and used in all the experiments described in this study.

Characterization of ATP (ADP)-Binding—The *M. avium* DnaA protein contains a Walker type ATP binding motif, indicating that it binds and possibly hydrolyzes ATP (*26, 27*). To determine *M. avium* DnaA protein binding to ATP, a nitrocellulose filter binding assay was performed. *M. avium* DnaA bound ATP at all temperatures tested, *i.e.* 0, 22, 30, and 37°C, and showed a pH optimum of 8.2 (data not shown). DnaA protein bound to ATP in a concentration-

dependent manner (Fig. 2) and binding reached saturation within five minutes of incubation (data not shown). Scatchard plot analyses revealed that K_D for ATP is 69 nM. Similar experiments were also carried out with ADP (Fig. 2B). *M. avium* DnaA protein bound ADP, much like ATP with a K_D value of 230 nM. The affinities of *M. avium* for ATP and ADP are found to be lower than those observed for the *E. coli* DnaA protein (*8*). The number of ATP binding sites per DnaA molecule was estimated to be 0.12, which is smaller than that of *E. coli* protein (*8*) and almost same as that of *B. subtilis* (*20*). The small number of binding sites may be due to a loss of activity during the preparation of the DnaA protein.

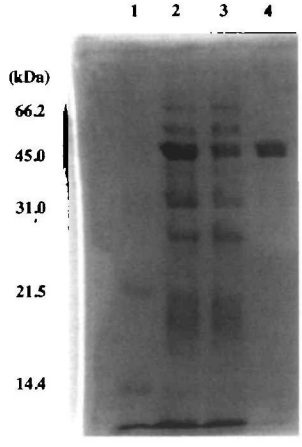

Fig. 1. **Isolation of *M. avium* DnaA protein.** Protein fractions from the Ni-column were applied to an SDS-polyacrylamide gel with a 10% separating gel and stained with Coomassie Brilliant Blue R-250. Lane 1, molecular mass markers; lane 2, total lysate; lane 3, flow-through fraction; lane 4, eluate (with 100 mM imidazole).

ATPase Activity—We detected the ATPase activity of DnaA protein by PEI–thin layer chromatography (TLC). Preliminary results indicated that it exhibits a weak ATPase activity that is temperature- and pH-dependent [data not shown]. Under optimal experimental conditions, *i.e.* Tris buffer, pH 8.2, at 37°C, the DnaA protein showed a slow turnover rate of 2.3×10^{-4} (s^{-1}). The ATPase activity appeared to be stimulated by *oriC*, *i.e.* the turnover rate in the presence of *oriC* DNA was 3.3×10^{-4} (s^{-1}). It should be noted that the DnaA proteins of both *E. coli* and *B. subtilis* hydrolyze ATP slowly (*8, 20*). The slow turnover rate suggests that either the *M. avium* DnaA ATPase activity is intrinsically low and or that the protein retains ADP following the hydrolysis of ATP. To understand this process further, DnaA-ATP complexes in both the presence and absence of *oriC* were prepared on ice, and the reaction samples were transferred to 37°C. At various periods after the start of incubation, samples were removed and the bound nucleotides were extracted and analyzed by TLC. As can be seen, a slow, time-dependent decrease in ATP with a concomitant increase in bound ADP were noted (Fig. 3). Hydrolysis of the tightly bound ATP in the presence of *oriC* was 50% complete in about 23 min whereas in the absence of *oriC*, 50% hydrolysis was achieved in about 38 min. Together, these results suggest that the *M. avium* DnaA pro-

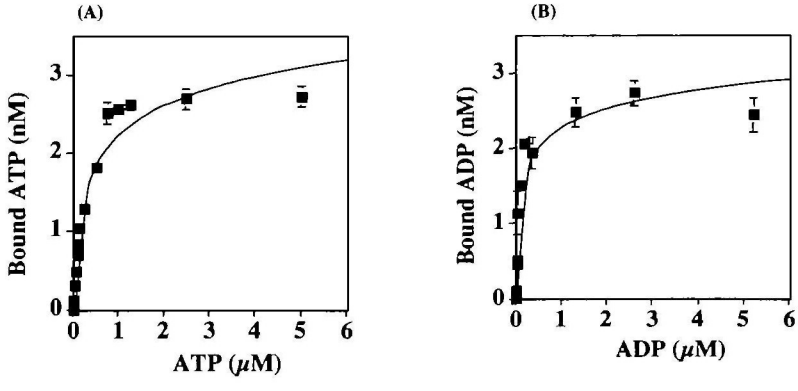

Fig. 2. **Binding of ATP or ADP to DnaA protein.** (A) DnaA protein (1.0 pmol) was incubated with [α-^{32}P]ATP (100 pmol) in 100 μl of E-buffer at 37°C for 15 min. Samples were collected on a nitrocellulose membrane and the amount of the radioactivity retained was measured in a liquid scintillation counter. (B) DnaA protein (7.0 pmol) was incubated with [^{14}C]ADP (700 pmol) in 700 μl of E-buffer and the bound ADP was determined as described above.

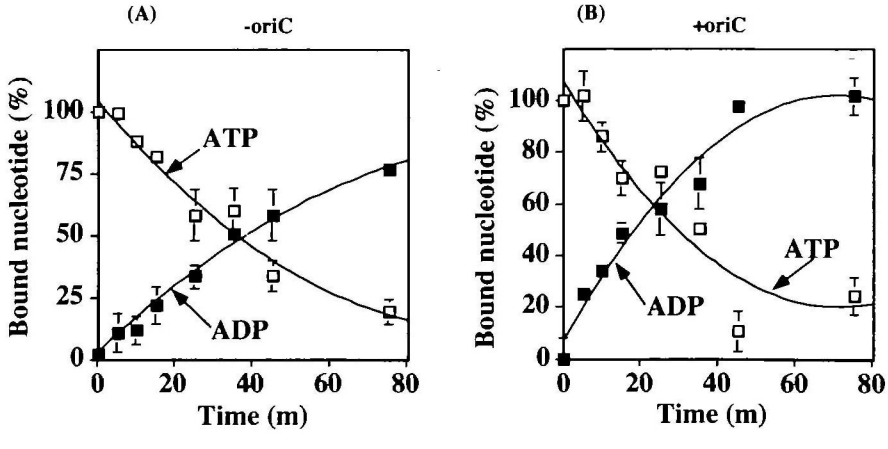

Fig. 3. **Time course of the hydrolysis ATP bound to DnaA protein.** DnaA protein (40 pmol) was incubated in E-buffer (40 μl) at 0°C for 15 min with [α-^{32}P]ATP (100 pmol), and then at 37°C with or without *oriC* DNA. Samples were filtered on nitrocellulose membranes and extracted with 50 μl of 1 M HCOOH. Samples (0.5 μl) were spotted on thin layer PEI-cellulose plates. Chromatography was with 1 M HCOOH–0.4 M LiCl. Open and closed squares represent bound ATP and ADP, respectively.

tein has a weak ATPase activity and remains bound to ADP following hydrolysis of ATP. Furthermore, *ori*C stimulates the ATPase activity.

The Dissociation of ADP from DnaA Protein—Our results showing comparable binding affinities of DnaA for both ATP and ADP (Fig. 2) combined with the weak ATPase activity (Fig. 3) suggest that the exchange of ATP for bound ADP is feeble. To test this prediction, DnaA-ADP complexes were prepared and then challenged with ATP. As can be seen, dissociation of ADP from DnaA protein in the presence of excess ATP proceeded only half way in about 60 min (Fig. 4). Under the same conditions, in the absence of any added ATP, little or no ADP dissociated (Fig. 4). Together these results suggest that the dissociation of ADP from the *M. avium* DnaA is very slow and that the exchange of ATP for bound ADP is feeble. Presumably, this process could be aided by some cofactors (see below).

We noted that during purification the majority of the recombinant *M. avium* DnaA protein was found in the inclusion bodies and associated with membrane fractions. Considering the roles and action of phospholipids on *E. coli* DnaA protein-adenine nucleotide interactions, we wanted to test whether phospholipids also promote the dissociation of bound ADP from the *M. avium* DnaA protein. Individual phospholipid vesicles, phosphatidylinositol, cardipolipin, phosphatidylethanolamine, and phosphatidylglycerol, were prepared and incubated with the DnaA-ADP complexes for different periods of time to monitor ADP dissociation (Fig. 5). As can be seen, all acidic phopsholipids, namely phosphatidylinositol, cardipolipin and phosphatidylglycerol, promoted the dissociation of ADP, whereas the neutral phospholipid, phosphatidylethanolamine, did not (Fig. 5). Among the phospholipids, we found that phosphatidylinositol was more effective than phosphatidylglycerol and cardiolipin in promoting the dissociation of ADP. In the absence of any added phospholipid, the nucleotide-protein complex remained intact for at least 60 min. The time course for the dissociation of bound ADP by phospholipids revealed that the majority of the bound ADP dissociated in about 15 min, and that phosphatidylinositol was more effective than cardiolipin and phosphatidylglycerol (Fig. 6). We also tested the effect of *ori*C on phosphatidylinositol mediated ADP dissociation. We found that *ori*C plasmid stimulated the dissociation of ADP from DnaA, whereas control plasmid lacking the *M. avium ori*C insert did not (Fig. 7).

Phosphatidylinositol, which is an essential phospholipid in eukaryotic cells, has seldom been found in prokaryotic cells. The distribution of phosphatidylinositol in prokaryotes seems to be confined to some actinomycetes (*Mycobac-*

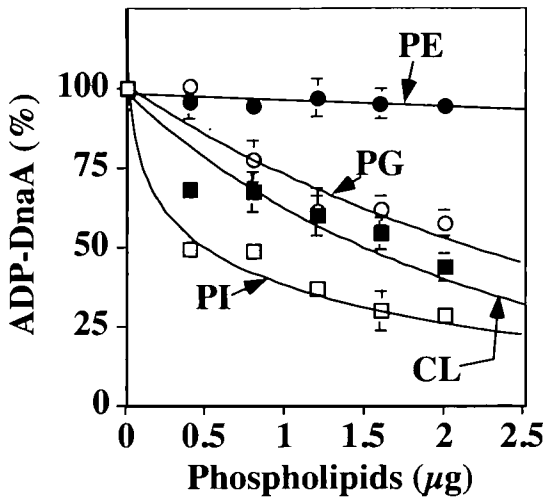

Fig. 5. **Dissociation of ADP from DnaA by phospholipids.** The DnaA-ADP complex was formed by incubating DnaA protein (10 pmol) with [^{14}C]ADP (732 pmol) at 0°C for 15 min. After exposure to various concentrations of phospholipids at 37°C for 15 min, the samples were filtered and processed as described above. PE (closed circles), phosphatidylethanolamine; PG (open circles), phosphatidylglycerol; PI (open squares), phosphatidylinositol; CL (closed squares), cardiolipin.

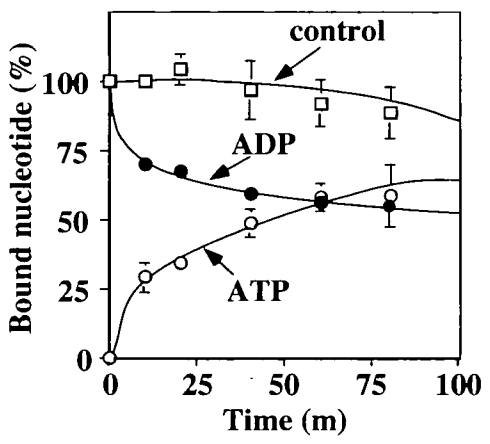

Fig. 4. **Exchange of ATP with ADP bound to DnaA protein.** DnaA protein (2.0 pmol) was incubated with 200 pmol of [^{14}C]ADP in E-buffer (100 μl) at 0°C for 15 min, and then with 1.0 mM [α-^{32}P]ATP at 37°C. The samples were filtered on membranes at the indicated time periods. The amounts of ATP (open circles) and ADP (closed circles) bound to DnaA protein were plotted. Control (open squares) refers to the reactions incubated in the absence of any added ATP.

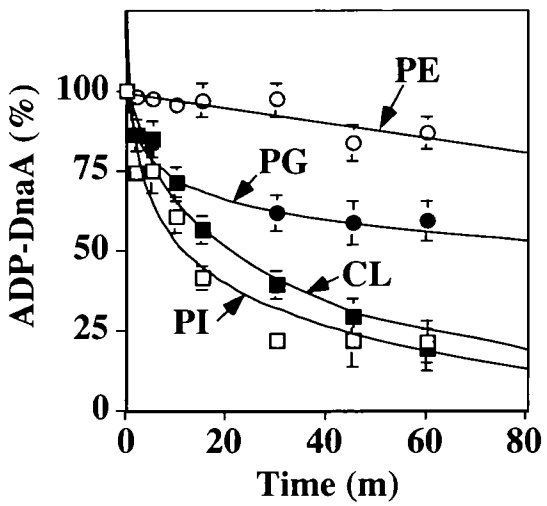

Fig. 6. **Time course of the dissociation of ADP by phospholipids.** The DnaA-ADP complexes as described above were exposed to phospholipids including PI (open squares), CL (closed squares), PE (open circles), PG (closed circles) at 37°C. At the indicated time periods, samples were removed, filtered on membranes and processed as described above.

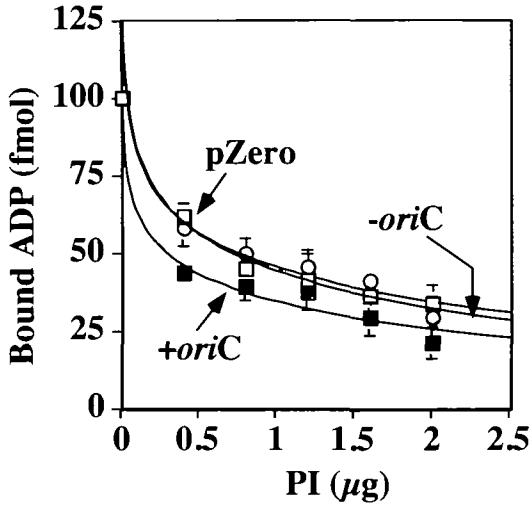

Fig. 7. **Effect of *ori*C on ADP dissociation.** DnaA (10 pmol) was incubated with [^{14}C]ADP (732 pmol) at 0°C for 15 min in a reaction volume of 100 μl. At the end of incubation, either *ori*C plasmid (0.05 μg) or control plasmid lacking the *ori*C insert (0.05 μg) and the indicated concentrations of PI were added. Samples were transferred to 37°C and incubation was continued for 15 min prior to processing as described above.

terium, Corynebacterium, Nocardia, Micromonospora, Streptomyces, and *Propionibacterium*) (reviewed in Ref. *28*). Phosphatidylinositol is one of most abundant mycobacterial phospholipids (*28*), and is an important intermediate in the generation of phopshatidylinositolmannosides and lipoarabinomannan (*29, 30*). The latter compound is a key mycobacterial lipoglycan and is believed to possess immunoregulatory properties. It remains to be tested whether the *M. avium* DnaA protein exhibits any preferential affinity for phosphatidylinositol.

This is the first report describing the preliminary biochemical characterization of *M. avium* DnaA protein. Comparative sequence analyses revealed that DnaA protein is structurally conserved in all eubacterial species that have been examined (*23, 26, 31, 32*). Our studies suggest the *M. avium* DnaA protein shares similarities with its *E. coli* counterpart. As reviewed, *E. coli ori*C replication is dependent on the nucleotide bound state of the DnaA protein, and acidic phospholipids rejuvenate moribund DnaA to an active form competent for replication reinitiation (*19*). The biochemical aspects of the initiation of replication in *M. avium* and other members of mycobacteria are unknown. We suggest that the initiation of *ori*C replication in *M. avium* involves intimate interactions among DnaA, adenine nucleotides, and phospholipids, much as in its *E. coli* counterpart. It should be noted that the sequence organization of the *M. avium ori*C is much more complex than that of the *E. coli ori*C (*23*). For example, the *M. avium ori*C contains 17 DnaA boxes that have little or no sequence similarity to the five DnaA boxes of *E. coli ori*C. Furthermore, *M. avium ori*C lacks any defined A-T rich repeat–like sequences as are found in the *ori*C of *E. coli*. In addition to the differences in *ori*C organization, *M. avium* and other mycobacterial members contain complex lipid-rich cell walls and exhibit varied growth rates. Considering the roles of acidic phospholipids on the regulation of *E. coli* DnaA activity (*19*) and the affinity of DnaA protein to the membranes (*18*), we envision that the precise mechanism, and possibly the regulation, of *M. avium ori*C replication may differ from that known for *E. coli*. Detailed investigations of the *M. avium* DnaA activities will provide insights into its *ori*C replication process.

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