Electrostatic Interactions during Acidic Phospholipid Reactivation of DnaA Protein, the *Escherichia coli* Initiator of Chromosomal Replication†

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ABSTRACT: The initiation of *Escherichia coli* chromosomal replication by DnaA protein is strongly influenced by the tight binding of the nucleotides ATP and ADP. Anionic phospholipids in a fluid bilayer promote the conversion of inactive ADP-DnaA protein to replicatively active ATP-DnaA protein in vitro, and thus likely play a key role in regulating DnaA activity. Previous studies have revealed that, during this reactivation, a specific region of DnaA protein inserts into the hydrophobic portion of the lipid bilayer in an acidic phospholipid-dependent manner. To elucidate the requirement for acidic phospholipids in the reactivation process, the contribution of electrostatic forces in the interaction of DnaA and lipid was examined. DnaA−lipid binding required anionic phospholipids, and DnaA−lipid binding as well as lipid-mediated release of DnaA-bound nucleotide were inhibited by increased ionic strength, suggesting the involvement of electrostatic interactions in these processes. As the vesicular content of acidic phospholipids was increased, both nucleotide release and DnaA−lipid binding increased in a linear, parallel manner. Given that DnaA−membrane binding, the insertion of DnaA into the membrane, and the consequent nucleotide release all require anionic phospholipids, the acidic headgroup may be necessary to recruit DnaA protein to the membrane for insertion and subsequent reactivation for replication.

DnaA protein initiates *Escherichia coli* chromosomal replication by binding to four 9-mer sequences within the unique origin, *oriC*, to form an initial complex. Approximately 20 DnaA monomers bind cooperatively to *oriC* to form a large nucleosome-like complex that encompasses approximately 200 base pairs of negatively supercoiled DNA (*1, 2*). In the presence of architectural proteins HU or IHF,[1] ATP, and sufficient temperature, DnaA protein promotes strand opening of three tandemly repeated AT-rich 13-mers in *oriC* to form an open complex (*3, 4*). It is this complex that gives rise to the two replication forks containing the proteins necessary for DNA replication (*5*).

The initiation activity of DnaA protein is strongly influenced in vitro by the tight binding of the nucleotides ATP and ADP (*6*). In its ATP form, DnaA protein is active for initiating replication. Slow DNA-dependent hydrolysis of the bound ATP to ADP, however, converts ATP-DnaA into the ADP form, which is unable to promote strand opening (*3, 6*−*8*). Even in the presence of high concentrations of ATP, reactivation of ADP-DnaA through exchange of ATP for bound ADP is extremely slow (*6*). Therefore, a mechanism responsible for modulating the state of DnaA protein between its active ATP form and its inactive ADP form is likely to play an important role in the regulation of chromosomal replication in *E. coli*.

Two possible targets for such a regulatory mechanism are (i) the slow hydrolysis of ATP to ADP, which renders DnaA protein replicatively inactive, and (ii) the exchange of ATP for ADP which reactivates DnaA protein for replication. Related to the first, recent work suggests that the β-subunit of DNA polymerase III, when assembled as a sliding clamp, is responsible for negatively regulating DnaA protein by accelerating the hydrolysis of the bound ATP to ADP (*9*).

Toward the second mechanism, results from both in vivo and in vitro studies implicate acidic phospholipids in the rejuvenation of replicatively feeble ADP-DnaA. In the presence of *oriC*, anionic phospholipids in a fluid bilayer promote the in vitro exchange of ATP for ADP, and thereby reactivate the initiation function of DnaA protein (*10*−*13*). In vivo, disrupted expression of the gene encoding phosphatidylglycerol synthase results in cells that are depleted of acidic lipids, and thus, arrested for growth (*14*). The normal requirement for DnaA-dependent initiations at *oriC* can be bypassed in cells having a specific genetic background (*recA*$^+$, *rnhA*$^-$) that permits initiations to occur at sites elsewhere on the chromosome (a process known as constitutive stable DNA replication, cSDR). Growth-arrest of cells unable to synthesize acidic phospholipids is relieved when the cells are also able to carry out cSDR (*15*), providing a strong link between cellular acidic phospholipids and DnaA-dependent initiation of chromosomal replication.

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[1] Abbreviations: IHF, integration host factor; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria broth; SOPG, 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicle.

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Despite the evidence for membrane participation in the control of DnaA protein activity, the physical interactions involved in this regulation have only recently begun to be defined. The requirement for fluidity (*11, 13*) suggests that reactivation may involve membrane insertion. In fact, recent experiments utilizing a photoactivatable phospholipid analogue revealed that a specific region of DnaA protein inserts into the hydrophobic region of the membrane bilayer. This insertion is accompanied by membrane-mediated release of nucleotide from DnaA protein and, like nucleotide release, is only efficient with membranes composed of acidic phospholipid and at temperatures sufficient for a fluid phase (*16*).

While the correlation of nucleotide release with membrane insertion is consistent with the observed requirement for fluidity in rejuvenation, the role of the acidic headgroup remains unclear. For a number of proteins known to be regulated through membrane interaction, an acidic headgroup is required for the electrostatic recruitment of the proteins to the lipid bilayer. A functional conformational change in *E. coli* SecA, which is involved in protein translocation, is promoted by hydrophobic insertion into acidic phospholipid-containing membranes (*17*). CTP:phosphocholine cytidylyltransferase, an enzyme involved in phosphatidylcholine synthesis in animal cells, is active only when membrane bound (*18*). The interaction of this enzyme with the membrane involves a two-step mechanism of electrostatic recruitment, followed by hydrophobic insertion into the bilayer (*19, 20*). In addition, human Src protein, which is active in its membrane-bound form (*21, 22*) is targeted to the membrane via both electrostatic binding of its basic domain to acidic membrane phospholipids and the hydrophobic insertion of a myristoyl group (*23*).

Because insertion of DnaA protein is dependent on acidic phospholipids, it is possible that the acidic headgroup is required, as with these other proteins, for the electrostatic recruitment of DnaA protein to the membrane. Recent experiments, in fact, have suggested an electrostatic requirement for productive DnaA-membrane interaction. Acidic gangliosides, which have a headgroup structure considerably different than that of glycerophospholipids, are efficient in promoting nucleotide release from DnaA protein, suggesting that it is the acidic surface created by these molecules that is important (*24*). Here, by direct measurement of DnaA−membrane binding using flotation gradient ultracentrifugation and DnaA−lipid interactions with intrinsic tryptophan fluorescence, the involvement of electrostatic forces in productive DnaA−membrane interactions has been examined.

EXPERIMENTAL PROCEDURES

Materials. Sources were as follows: 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)], Avanti Polar Lipids, Inc.; L-3-phosphatidylcholine, 1,2-di-[1-^{14}C]palmitoyl (118 Ci/mol), Amersham Life Science Products; trans-[^{35}S]-label, ICN; [α-^{32} P]ATP, NEN Life Science Products; type HA nitrocellulose filters, Millipore Corp.; Probond resin, Invitrogen.

His-[^{35}S]DnaA Purification. Recombinant metabolically labeled polyhistidine-tagged DnaA protein was purified from BL21(DE3)pLysE transformed with pZL411, a high expression plasmid encoding polyhistidine-tagged DnaA (*36*). A single transformed colony was used to inoculate Luria broth (LB; 5 mL) or M9 minimal medium (*25*) (10 mL) lacking cysteine and methionine, and the cultures were incubated up to 14 h (37 °C). Overnight cultures were diluted into either LB medium (100 mL) or M9 minimal medium (300 mL) to an optical density (OD_{600}) of 0.05. At an OD_{600} of 0.4−0.5, the LB culture was supplemented with IPTG to 1 mM, and DnaA expression induced for 1.5 h at 37 °C. At an OD_{600} of 0.25, the M9 culture was supplemented with IPTG to 1 mM, and DnaA expression induced for 15 min (37 °C), followed by addition of trans-label (28 mCi; >1000 Ci/mmol) and DnaA expression continued for 2 additional hours (37 °C). Induced cells were harvested by centrifugation (5000*g*, 10 min, 4 °C). Cell pellets were resuspended in binding buffer [0.5−0.8 mL; 20 mM sodium phosphate (pH 7.8), 500 mM NaCl, 5 mM imidazole], flash frozen in liquid N_2 and stored at −80 °C. Frozen cells were thawed on ice followed by the addition of lysozyme (300 μg/mL, 20 min, on ice). Lysozyme-treated cells were subjected to four cycles of freeze−thawing (liquid N_2 and 37 °C water bath). Dnase I (40 μg/mL) and $MgCl_2$ (10 mM) were added, and the mixtures were maintained briefly on ice (5 min). The lysates from the M9 and LB cultures were pooled and clarified by centrifugation (100000*g*, 40 min, 4 °C). The following steps for purification of His-DnaA were carried out at 4 °C. The clarified cell lysate was continually mixed by gentle rotation (30 min) with Probond resin (200 μL) equilibrated in binding buffer. Nonspecifically bound proteins were removed by washing the resin with wash buffer A [volume equal to 15 times the resin volume; 20 mM sodium phosphate (pH 7.8), 500 mM NaCl, and 100 mM imidazole], followed by washing the resin with wash buffer B [volume equal to 15 times the resin volume; 20 mM sodium phosphate (pH 7.8), 500 mM NaCl, 100 mM imidazole, 0.1% (w/v) β-octylglucoside]. Residual detergent was removed by washing the resin with 30 resin vol of wash buffer A. Additional contaminants were removed by treatment with 7 M urea in binding buffer (1 mL, 40 min). Residual urea was removed by washing with 15 resin vol of buffer HD [50 mM PIPES•KOH (pH 6.8 at 1 M), 10 mM magnesium acetate, 200 mM ammonium sulfate, 20% (w/v) sucrose]. Polyhistidine-tagged DnaA protein was eluted with buffer HD that contained imidazole (1 M). Elution fractions were dialyzed against buffer HD containing EDTA (0.1 mM) and dithiothreitol (2 mM) (500 mL, four buffer changes, 30 min each). Aggregated proteins were removed from the soluble fraction by centrifugation (100000*g*, 20 min).

Vesicle Preparation. Total *E. coli* phospholipids, stearoyl-oleoyl phosphatidylglycerol (SOPG), stearoyl-oleoyl phosphatidylcholine (SOPC), or mixtures of SOPG and SOPC in chloroform were dried under a stream of nitrogen gas and resuspended in water by sonication (30 min, 0 °C) with bursts (30−50%) using a microtip sonicator (Rx Technologies, Inc.). Sonicated lipids were centrifuged (20 min, 140000*g*, 22 °C), and the supernatants that contained small unilamellar vesicles (SUVs) were collected. Phospholipid concentrations were determined by assaying for total phosphate (*26*).

Lipid Migration. SOPG/SOPC (4:1 molar ratio), SOPC, or *E. coli* SUVs were prepared as described above, with the inclusion of L-3-phosphatidylcholine, 1,2-di[1-^{14}C] palmitoyl

(118 Ci/mol) as a tracer. SUVs (15 nmol of lipid) were incubated with ATP-DnaA (45 pmol) in buffer C [final volume of 175 μL; 50 mM Tricine-KOH, pH 8.25 at 1 M; 2.5 mM magnesium acetate; 0.323 mM EDTA; 8 mM dithiothreitol; 20% (v/v) glycerol] which also contained 30% (w/v) sucrose. The mixture was placed in the bottom of a polyallomer ultracentrifuge tube, with steps (600 μL each) of buffer C containing 20, 15, and 10% sucrose (w/v) layered on top. The gradients were centrifuged (16 h, 38 °C, 215000*g*) and harvested in steps from the top (four 425 μL fractions, followed by one 275 μL fraction). Migration of lipid was assessed by liquid scintillation counting of the fractions; radiolabel found in the top four fractions represented migrated lipid.

DnaA−Lipid Binding. Phospholipid vesicles and ATP-DnaA protein or the ATP form of His-[^{35}S]DnaA protein were incubated (10 min, 38 °C; final volume of 175 μL) in buffer C containing 30% (w/v) sucrose. The mixture was subjected to flotation gradient ultracentrifugation as described in *Lipid Migration*, and DnaA protein was detected by either liquid scintillation counting or immunoblot analysis of harvested fractions. DnaA protein in the harvested fractions was considered soluble, while that remaining on the walls of the gradient tube as insoluble. DnaA protein found in the top four fractions was considered as phospholipid-bound.

Nucleotide Binding and Phospholipid-Mediated Nucleotide Release. His-[^{35}S]DnaA protein in buffer D [50 mM Tricine-KOH, pH 8.25, at 1 M; 2.5 mM magnesium acetate; 0.323 mM EDTA; 8 mM dithiothreitol; 0.0065% (v/v) Triton X-100; 20% (v/v) glycerol] was mixed with [α-^{32} P]ATP (1 μM) to produce ATP-DnaA protein. Nucleotide-bound DnaA protein was then incubated (10 min, 38 °C) with the indicated amounts of phospholipid. Retained nucleotide was quantitated by filtration of samples through nitrocellulose filters presoaked in buffer G [50 mM Tricine-KOH, pH 8.25 at 1 M; 0.5 mM magnesium acetate; 0.3 mM EDTA; 5 mM dithiothreitol; 0.005% (v/v) Triton X-100; 10mM ammonium sulfate; 17% (v/v) glycerol]. Filters were washed with buffer G (5 mL), dried, and the retained nucleotide measured by scintillation counting, with the background contribution of radiolabel from His-[^{35}S]DnaA being subtracted. Nucleotide binding (100%) corresponds to the amount of ATP bound by DnaA protein when treated (10 min, 38 °C) in the absence of phospholipids.

Measurement of Ionic Strength. The ionic strength, *I*, was measured as $I = \Sigma c_i z_i^2/2$ where c_i is the molar concentration of the ionic species, *i*, of charge z_i.

Fluorescence Measurements. The tryptophan fluorescence of DnaA protein was measured with an SLM AB2 luminescence spectrometer (SLM Instruments, Inc., Urbana, IL) in a 1 cm cuvette. Emission spectra were acquired at 38 °C with an excitation at 290 nm and emission and excitation slit widths at 4 nm. The signal was corrected for variations in intensity by using a reference channel. Fluorescence emission spectra were obtained for ATP-DnaA protein (98 pmol) in buffer C or buffer C containing 0.75 M KCl (500 μL). SOPG/SOPC (4:1) or SOPC SUVs (40 nmol) were then added and the fluorescence emission spectra were recorded after 2 min. The fluorescence spectra obtained after 2 min were similar to those obtained after 10 min (data not shown). DnaA spectra were corrected by subtraction of the spectra of buffer C with or without lipid.

Other Methods. Nontagged DnaA protein was purified (*27*) from BL21(DE3)pLysS/pKA211, a strain of *E. coli* transformed with a high level DnaA expression plasmid under the control of a bacteriophage T7 RNA polymerase promoter (T. Katayama, unpublished data). *E. coli* phospholipid extraction was performed as described by Kagawa and Racker (*28*) from strain W3110 [F$^-$, λ$^-$, IN(*rrnD-rrnE*)]. Protein quantitation was performed as described by Bradford (*29*). Immunoblots were developed with Vistra ECF substrate (Amersham), and chemiluminescence was analyzed with a STORM 840 (Molecular Dynamics) and ImageQuant software (Molecular Dynamics).

RESULTS

Binding of DnaA to Acidic versus Neutral Phospholipid Vesicles. Previous experiments demonstrated that a portion of DnaA protein inserts into acidic, but not neutral membranes (*16*). The failure to insert into neutral vesicles could be caused by either (i) an inability to insert into the hydrophobic interior of the bilayer even though DnaA is bound to the polar surface, or (ii) an overall failure of DnaA to associate with neutral membranes due to the absence of anionic headgroups. To distinguish between these two possibilities, the binding of DnaA protein to lipid bilayers was examined using flotation gradient centrifugation. Lipid loaded at the bottom of an appropriate density gradient floats toward the top during high-speed centrifugation; any protein associated with the lipid will also migrate upward. Under the conditions used here, 80−85% of either acidic small vesicles composed of the 1-stearoyl-2-oleoyl forms of phosphatidylglycerol and phosphatidylcholine (SOPG:SOPC, 4:1) or neutral vesicles composed of 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) migrated from the bottom of the gradient to the top four gradient fractions (Figure 1, solid bars). To evaluate the extent that DnaA was able to stably bind to the vesicles, ATP-DnaA was incubated with neutral (SOPC) or acidic vesicles (SOPG:SOPC, 4:1). The molar ratio of lipid to protein (333:1) was one that also results in maximum release of DnaA-bound nucleotide if the vesicles are acidic in nature (data not shown). The mixtures were subjected to flotation gradient centrifugation and the location of DnaA within gradient fractions was determined by immunoblot analysis (Table 1). As expected, in the absence of lipid, DnaA protein remained at the bottom of the gradient (Table 1, line 1), while only a small percentage of DnaA was found associated with neutral phospholipids (Table 1, line 3). In contrast, a large percentage of DnaA protein bound to acidic vesicles and migrated upward (Table 1, line 2).

Because quantitating protein levels with immunoblots can be variable, a metabolically radiolabeled histidine-tagged form of DnaA protein, His-[^{35}S]DnaA, was also used to test for lipid binding. Histidine-tagged DnaA binds nucleotide with the same affinity, initiates DNA replication in a reconstituted system to the same extent, and retains the same specificity for acidic phospholipids in nucleotide release as wild-type DnaA protein (Z.L. and E.C., manuscript submitted for publication), and thus may serve as a useful tool for evaluating DnaA−lipid binding. As was done with nontagged DnaA protein, His-DnaA was mixed with lipids at a molar ratio (225:1; lipid:DnaA) which results in nearly maximal nucleotide release (Figure 2A). His-DnaA demonstrated similar specificity as nontagged DnaA for binding to acidic,

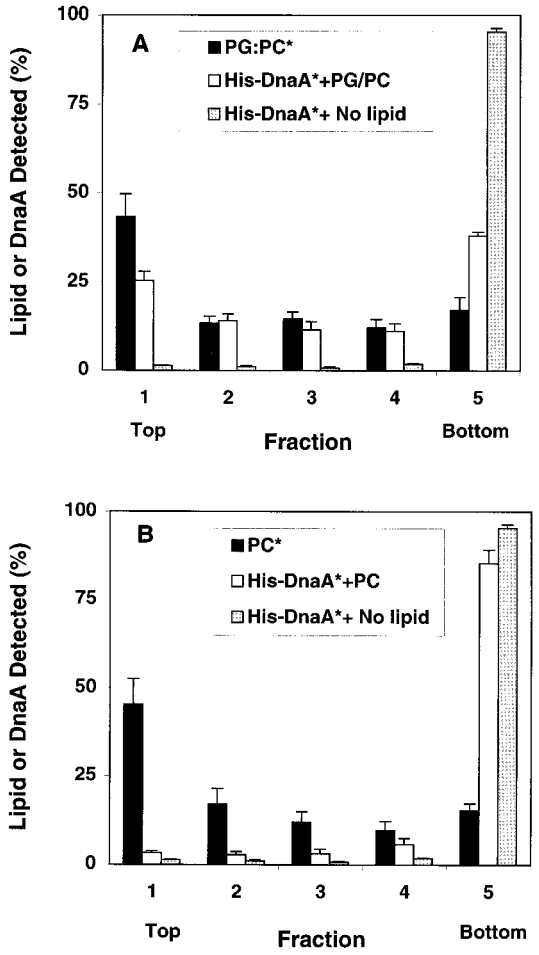

FIGURE 1: Distribution of SUVs and DnaA protein within flotation gradients. Lipid migration: (A) SOPG/SOPC (4:1) or (B) SOPC SUVs (4.5 nmol) containing [^{14}C]DPPC as a tracer, were subjected to flotation gradient centrifugation, and lipid within harvested fractions was detected by liquid scintillation counting (see Experimental Procedures). DnaA migration (A and B): His-[^{35}S]DnaA-ATP protein (45 pmol) was subjected to flotation gradient centrifugation and protein in harvested fractions detected by liquid scintillation counting. DnaA−lipid binding: (A) SOPG/SOPC (4:1) or (B) SOPC SUVs (4.5 nmol) were incubated (10 min, 38 °C) with His-[^{35}S]DnaA-ATP (20 pmol) and subjected to flotation gradient centrifugation. Gradient fractions were harvested and protein detected by liquid scintillation counting. (*) The radiolabeled species being measured. All values are an average of at least three independent experiments, with the vertical bars representing standard deviations.

but not neutral membranes (Figure 1, open bars; and Table 1, lines 5 and 6), demonstrating that tagged DnaA is representative of nontagged DnaA also with respect to lipid binding. Additionally, His-DnaA, like nontagged DnaA, remained at the bottom of the gradient (Figure 1, gray bars; and Table 1, line 4). Accurate quantitation of His-DnaA bound to lipids, achieved by scintillation counting of His-[^{35}S]DnaA in gradient fractions (Figure 1 and Table 1, lines 4−6), corroborate the results from the immunoblot analysis, making this the method of choice for measuring DnaA−lipid binding in subsequent experiments.

Membrane Composition Required for DnaA-Lipid Binding Parallels that Required for Nucleotide Release. To see if lipid-mediated nucleotide release correlates with membrane binding, a titration of both acidic and neutral SUVs was performed. The conditions for efficient DnaA−lipid binding

Table 1: DnaA Protein Binds to Acidic, but Not Neutral Phospholipid[a]

	migration (%) detected by:	
	Western analysis	scintillation counting
1. DnaA	2	ND
2. DnaA + PG/PC	76	ND
3. DnaA + PC	3	ND
4. His-DnaA	2	4.6 ± 1
5. His-DnaA + PG/PC	80	62 ± 1.3
6. His-DnaA + PC	3	15 ± 4.5

[a] Lines 1−3: SOPG/SOPC (4:1) or SOPC vesicles (15 nmol) were incubated (10 min, 38 °C) with DnaA-ATP (45 pmol) and subjected to flotation gradient centrifugation (see Experimental Procedures). Lines 4−6: SOPG/SOPC (4:1) or SOPC vesicles (4.5 nmol) were incubated (10 min, 38 °C) with His-[^{35}S]DnaA-ATP (20 pmol) and subjected to flotation gradient centrifugation. Gradient fractions were harvested and the protein detected by Western analysis or scintillation counting. Data values for analysis by scintillation counting are an average of three independent experiments, with standard deviations shown. ND, not determined.

paralleled those needed for nucleotide release; as the concentration of acidic lipid (SOPG) was increased, both nucleotide release and DnaA−lipid binding increased in a similar fashion. Likewise, titration of neutral phospholipid (SOPC) had little effect on both nucleotide release and DnaA−lipid binding (Figure 2).

To further investigate the requirement that lipid bilayers must have anionic headgroups to regulate DnaA activity, the level of acidic lipid (SOPG) in SUVs was varied (with the balance of vesicle lipid being SOPC) and the vesicles' ability to bind DnaA protein and promote release of DnaA-bound nucleotide was examined. As the mole percent of acidic lipid in the vesicles increased, both activities were affected similarly (Figure 3). Neither nucleotide release nor membrane binding exhibited a sharp threshold for acidic lipid, instead, each increased largely in a linear manner as the vesicles became more anionic.

Membranes of *E. coli* are composed of approximately 75% neutral and 25% acidic phospholipids (*30*). SUVs prepared with phospholipids extracted from *E. coli* are capable of promoting the release of nucleotide from DnaA protein (*2*, *10*−*12*) (Figure 4). The amount of *E. coli* lipid needed to induce maximal release of bound nucleotide is identical to that required for maximum DnaA binding to membranes (Figure 4). Interestingly, both nucleotide release by and DnaA binding to *E. coli* phospholipids correlated well with the extent of the effects that defined vesicles containing 20−30% acidic lipid had on those functions (Figure 3). The distribution of *E. coli* phospholipid vesicles and DnaA protein within the gradients (data not shown) was similar to that observed with both acidic and neutral vesicles (Figure 1).

Acidic but Not Neutral Membrane Vesicles Promote a Blue-Shift in DnaA Fluorescence Emission. As an independent means of probing DnaA−lipid interactions, the fluorescence of tryptophan residues of nontagged DnaA protein was monitored in the absence and presence of acidic and neutral membrane vesicles. The tryptophan emission spectrum undergoes a characteristic shift in the emission spectrum to a shorter wavelength when one or more residues transfers from an aqueous to a hydrophobic environment (*31*). One cause of such a shift would be the binding and/or insertion of a trytophan residue(s) of DnaA protein into membrane

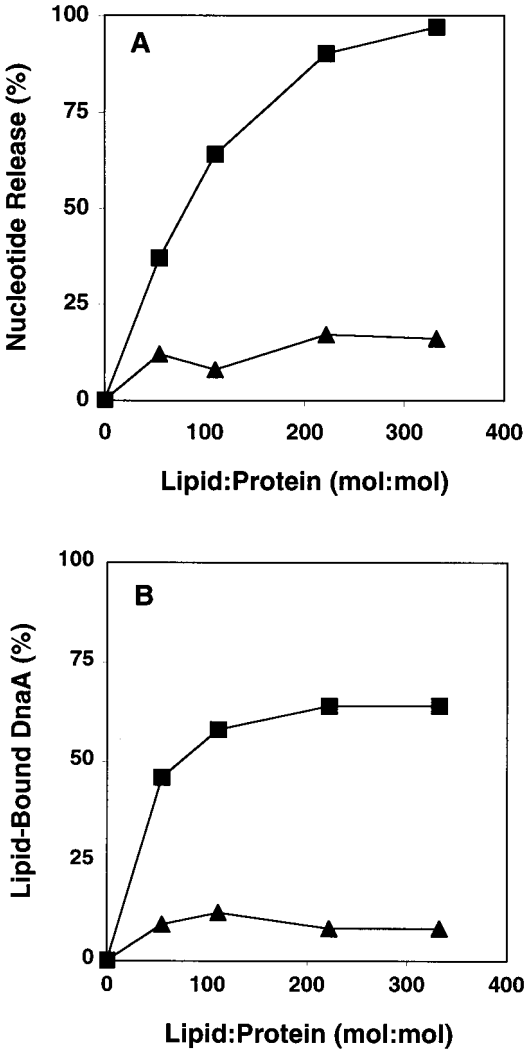

FIGURE 2: The ability of acidic versus neutral phospholipids to promote nucleotide release and bind DnaA protein. (A) Nucleotide release: SOPG/SOPC (molar ratio of 4:1) (■) or SOPC (▲) SUVs (0.1−0.6 nmol) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA (1.8 pmol) and retained ATP was measured by a filter retention assay. (B) DnaA−lipid binding: SOPG/SOPC (molar ratio of 4:1) (■) or SOPC (▲) SUVs (1−7 nmol) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA (20 pmol), subjected to flotation gradient centrifugation, and protein in gradient fractions detected by scintillation counting. Results are typical of three independently performed experiments.

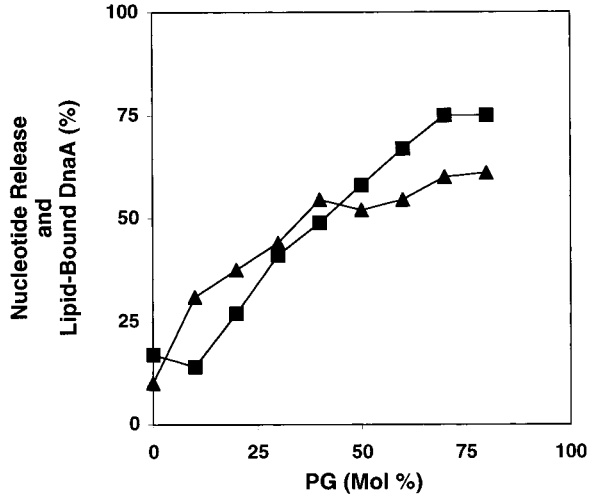

FIGURE 3: Increased vesicular content of acidic phospholipids enhances nucleotide release and DnaA−lipid binding. Nucleotide release (■): SUVs (0.4 nmol; 0−80% SOPG and 100−20% SOPC) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA protein (1.8 pmol) and retained ATP was measured by a filter retention assay. DnaA−lipid binding (▲): SOPG/SOPC SUVs (4.5 nmol; 0−80% SOPG and 100−20% SOPC) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA protein (20 pmol), subjected to flotation gradient centrifugation, and protein in gradient fractions was detected by scintillation counting. Results are typical of three independently performed experiments.

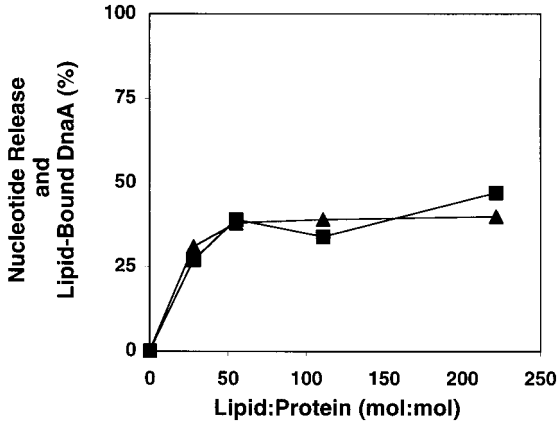

FIGURE 4: Nucleotide release and DnaA binding by *E. coli* phospholipid vesicles. Nucleotide release (■): *E. coli* phospholipid SUVs (0.05−0.4 nmol) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA protein (1.8 pmol) and retained ATP was measured by a filter retention assay. DnaA−lipid binding (▲): *E. coli* phospholipid SUVs (0.56−4.5 nmol) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA protein (20 pmol), subjected to flotation gradient centrifugation, and protein in gradient fractions was detected by scintillation counting. Results are typical of three independently performed experiments.

vesicles. At a molar ratio of lipid to protein (400:1), in which lipid is in slight excess for nucleotide release, the emission maximum of nontagged DnaA protein tryptophan fluorescence (344 nm) shifted 7 nm to 337 nm in the presence of acidic membrane vesicles. (Figure 5A). In contrast, addition of neutral membrane vesicles caused no change in the emission spectrum (Figure 5B). Thus, like nucleotide release, membrane insertion, and DnaA−lipid binding (as measured by gradient centrifugation), a transfer of one or more tryptophan residues to a hydrophobic environment is dependent on the presence of acidic phospholipid in membrane vesicles.

Increased Ionic Strength Inhibits Both Nucleotide Release and DnaA−Lipid Binding. The requirement for an acidic headgroup in lipid binding and nucleotide release suggests the involvement of electrostatic forces in productive DnaA−membrane interaction. To investigate this possibility, treatment of DnaA protein with acidic vesicles was carried out under conditions of varying ionic strength. As ionic strength increases, the negative surface potential of acidic membrane vesicles decreases, thus reducing electrostatic attractions that may exist between basic domains of a protein and the anionic headgroups of the lipid bilayer.

Increasing the ionic strength by KCl addition during the exposure of DnaA protein to acidic vesicles diminished, to a similar degree, the membrane binding capacity of DnaA and the ability of the lipids to promote release of DnaA-bound nucleotide (Figure 6A). Increased ionic strength also had a similar effect on nucleotide release and DnaA binding to *E. coli* phospholipid SUVs, with strong inhibition of both

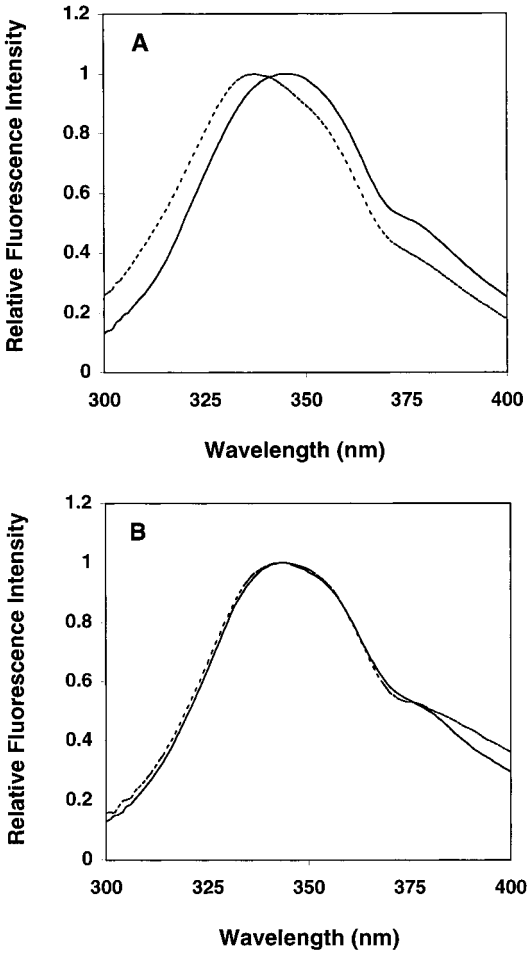

FIGURE 5: Peak normalized fluorescence spectra of DnaA protein in the presence of membrane vesicles. Emission spectra of DnaA protein in buffer (—), or DnaA protein in the presence (- - -) of acidic (A) or neutral membrane vesicles (B) were carried out as described in the Experimental Procedures.

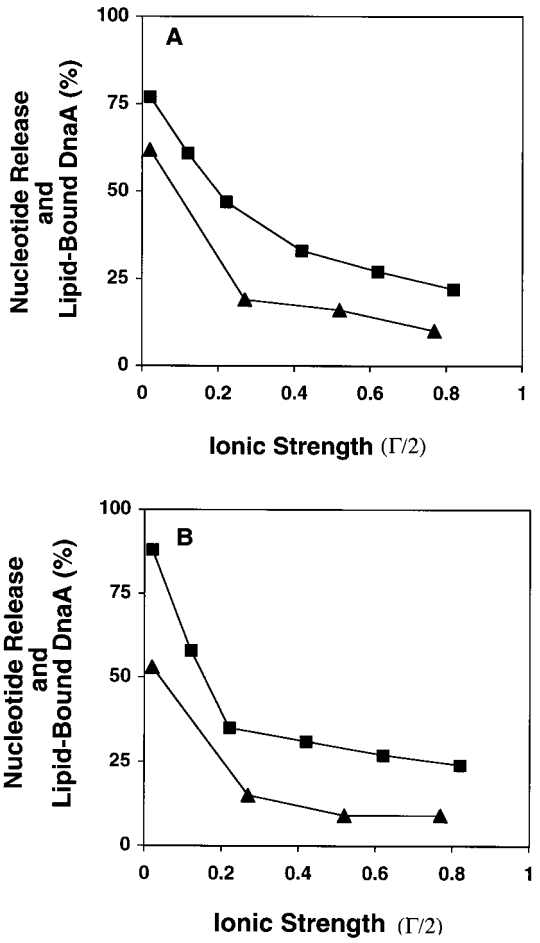

FIGURE 6: Increased ionic strength affects nucleotide release and DnaA−lipid binding. Nucleotide release (■): (A) SOPG/SOPC (molar ratio of 4:1) SUVs (0.4 nmol) or (B) *E. coli* phospholipid SUVs (1.2 nmol) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA protein (1.8 pmol) in buffer which was adjusted to an ionic strength of 0.02−0.75 with KCl. Retained ATP was measured by filter binding. DnaA−lipid binding (▲): (A) SOPG/SOPC (molar ratio of 4:1) SUVs (4.5 nmol) or (B) *E. coli* phospholipid SUVs (13.5 nmol) were incubated (10 min, 38 °C) with the ATP form of [^{35}S]His-DnaA protein (20 pmol) in a buffer adjusted to an ionic strength of 0.02−0.75 using KCl, subjected to flotation gradient centrifugation, and the protein in gradient fractions was detected by scintillation counting. Results are typical of three independently performed experiments.

activities at an ionic strength of 0.2−0.3 (Figure 6B). In contrast, increased ionic strength had no effect on the low level of nucleotide release or DnaA binding by neutral vesicles composed of phosphatidylcholine (data not shown). Additionally, increased ionic strength did not affect the migration of defined SOPG/SOPC vesicles or *E. coli* phospholipid vesicles, nor did it affect nucleotide binding to DnaA protein (data not shown). Identical concentrations of sodium glutamate inhibited both nucleotide release and lipid binding to a similar extent as KCl (data not shown), demonstrating that the observed inhibition is an effect of increased ionic strength rather than an effect mediated specifically by potassium or chloride ions.

Increased Ionic Strength Inhibits the Blue-Shift Promoted by Acidic Membrane Vesicles. The involvement of electrostatic forces in DnaA−lipid interactions was also examined by monitoring tryptophan fluorescence under conditions of varying ionic strength. Increasing the ionic strength in the DnaA−acidic lipid mixture inhibited the blue shift observed at low ionic strength (Figure 5A and Figure 7A), suggesting an inability of DnaA protein to interact with acidic membrane vesicles under these conditions. As expected, increased ionic strength had no effect on the emission spectrum of DnaA protein in the presence of neutral membrane vesicles (Figure 7B).

DISCUSSION

These studies, which examine the relationship between lipid-mediated release of DnaA-bound nucleotide and DnaA−membrane binding, support the hypothesis that bilayers must possess anionic headgroups for the electrostatic recruitment of DnaA protein to membranes. Like nucleotide release and membrane insertion, stable DnaA−lipid binding was efficient only with vesicles having acidic phospholipids. As the content of acidic lipid in vesicles was increased, both nucleotide release and DnaA−lipid binding were affected almost identically, showing a very tight link between stable membrane binding and release of bound nucleotide. Interestingly, a titration of *E. coli* phospholipid SUVs, which have a composition of approximately 25% acidic phospholipid, yielded nucleotide release and DnaA binding similar to those by defined lipid vesicles composed of 20−30% acidic lipid (cf., Figures 3 and 4). This suggests that the concentration of acidic lipid, rather than a specific *E. coli* phospholipid

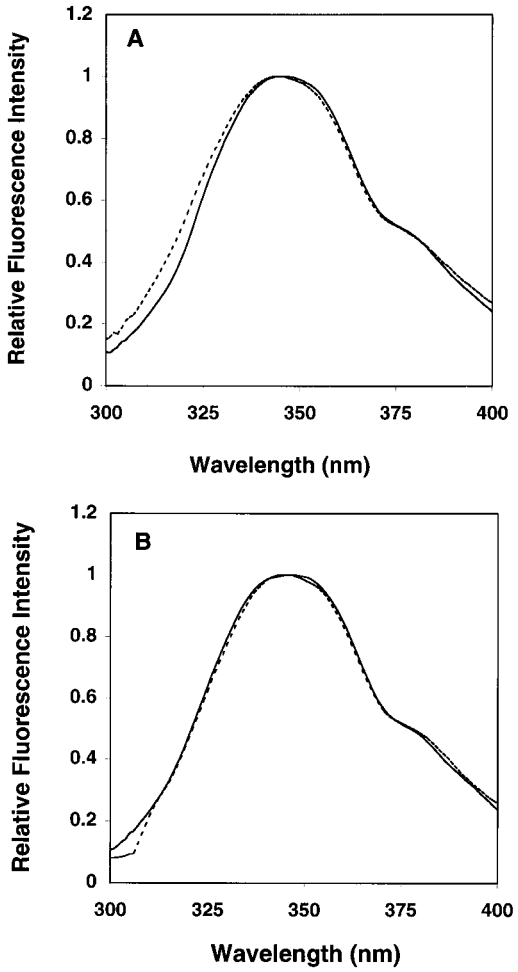

FIGURE 7: Peak normalized fluorescence of DnaA protein in the presence of membrane vesicles at increased ionic strength. Emission spectra of DnaA protein in buffer (—) or DnaA protein in the presence (- - -) of acidic (A) or neutral membrane vesicles (B) were carried out at an ionic strength of 0.75 as described in the Experimental Procedures.

component, is the important factor for productive DnaA−membrane interaction.

Previous studies suggested that interactions with acidic, but not neutral headgroups, may be responsible for nucleotide release. The possibility that productive association requires a specific headgroup conformation, and is independent of charge, could not be ruled out. Recognition of a required structure, in contrast to charge-dependent interactions, should act largely independent of the ionic strength of the environment. Here, however, we observed that both nucleotide release and DnaA binding by defined lipid vesicles and vesicles of *E. coli* phospholipids were significantly inhibited by increasing concentrations of a monovalent salt, implicating the involvement of electrostatic forces in functional DnaA−membrane interaction. The involvement of such forces is further supported by the absence of an effect of increased ionic strength on the low level of nucleotide release and lipid binding by neutral phospholipid vesicles (SOPC) (data not shown).

In addition to direct measurement of lipid binding, the interaction of DnaA protein with membranes was examined by following the changes in the fluorescence emission maximum of tryptophan residues upon exposure of DnaA protein to lipid vesicles. Monitoring such changes has been used to study the lipid interaction of a number of proteins, including pediocin PA-1 (*32*) and CTP:phosphocholine cytidylyltransferase (*33*). DnaA protein contains five tryptophan residues: W6, W25, W50, W117, and W288 (*34*). A blue-shift in the emission maximum, which is characteristic of the transition of one or more of these tryptophan residues to a hydrophobic environment, occurred only with vesicles containing acidic phospholipid. In addition, like nucleotide release and lipid binding, this shift was inhibited by increased ionic strength. Since it has been demonstrated that the region of membrane insertion is bounded by amino acids 309−399 of DnaA protein (*16*), W288 is a strong candidate for a tryptophan residue responsible for this effect. Alternatively, interaction with acidic membranes may promote a conformational change in DnaA protein that places one or more tryptophan residues within a hydrophobic domain of the protein itself. Regardless of the exact mechanism, the change in tryptophan environment was dependent upon acidic phospholipids.

These results have led us to propose a model for the membrane reactivation of ADP-DnaA protein (Figure 8). DnaA protein is recruited to the membrane via electrostatic

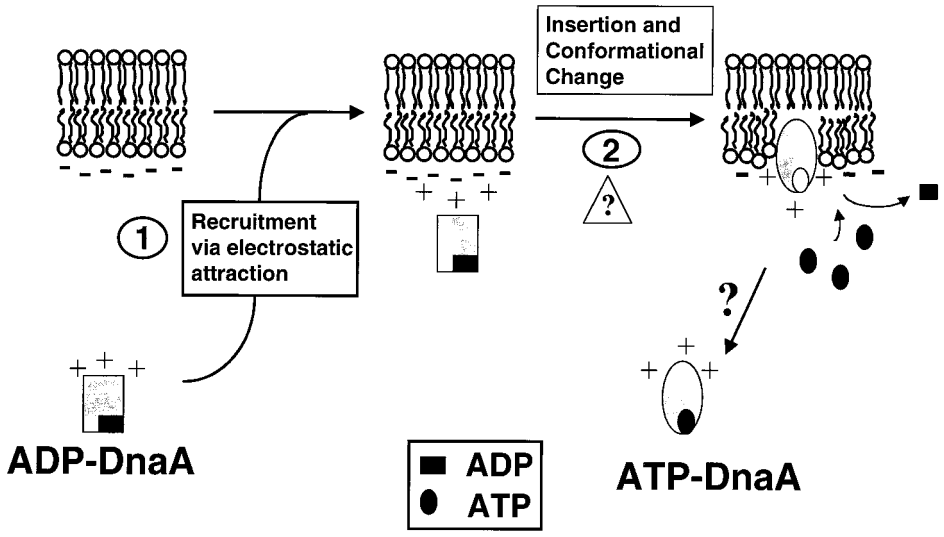

FIGURE 8: Regulation of DnaA protein activity through membrane interaction.

attractions between the negatively charged acidic headgroups of the membrane and basic regions of DnaA. By being localized at the bilayer surface, the apparent concentration of DnaA is raised, increasing the likelihood of insertion of a specific region of DnaA into the hydrophobic portion of the membrane. The insertion may result in a conformational change that allows for exchange of nucleotide and consequent reactivation of DnaA protein for replication. As is the case for nucleotide release (*10*, *11*), no distinctions have been observed between the lipid binding of ADP-DnaA versus ATP-DnaA (data not shown), suggesting that both nucleotide forms may be recruited to the membrane in a similar fashion. However, since the cellular concentration of ATP is 10−15-fold higher than that of ADP (3 mM and 250 μM, respectively), net formation of ATP-DnaA during membrane-mediated nucleotide exchange would be highly favored.

It is worth noting that within the region of DnaA protein that inserts into the membrane there is a putative amphipathic helix that may be involved in productive DnaA−membrane interaction (*24*, *34*). Determining which characteristics of this region are significant for membrane association is currently being aided by in vivo and in vitro analysis of mutant forms of DnaA protein, including those that contain single amino acid substitutions within the putative amphipathic helix region. Some of these are able to suppress arrested growth in an *E. coli* strain that cannot grow because acidic phospholipid synthesis has been turned off (unpublished data).

How the reactivation of DnaA protein is regulated still remains unanswered. Recruitment of DnaA protein to the membrane is a process that may well serve as a target for a regulatory mechanism; controlled productive binding of DnaA protein to the cytoplasmic membrane may be an essential element in the correct timing of the initiation of chromosomal replication. Perhaps related, acidic lipids can be induced to cluster within a bilayer when they possess a different number of alkyl chains than the host lipid. Interestingly, such membranes are more efficient at promoting nucleotide release than acidic membranes containing non-cluster inducing homoalkyl chains (*35*). It has been hypothesized that in *E. coli*, a cell-cycle-specific signaling event may activate a specific phospholipid modifying enzyme(s) that would give rise to membrane heterogeneity. A consequence would be acidic phospholipid clustering that stimulates nucleotide release from DnaA protein (*35*).

Other proteins may be a necessary component for the controlled binding of DnaA protein to the cytoplasmic membrane. Preliminary experiments with *E. coli* inverted inner membrane vesicles suggest the involvement of a peripheral membrane protein that negatively regulates lipid-mediated nucleotide release from DnaA (unpublished data). Such a protein may mediate its effect by inhibiting productive DnaA−membrane association. Immunogold labeling and immunofluorescence experiments with anti-DnaA IgG localize DnaA protein in close proximity to the plasma membrane during most of the cell cycle, suggesting that electrostatic recruitment alone may not be sufficient for the regulation of DnaA protein (Newman and Crooke, unpublished data).

Little is known about the role of *oriC* in this process. In vitro, the rejuvenation of the ADP form of DnaA protein to ATP-DnaA is *oriC* dependent (*10*−*12*). Photolabeling of DnaA protein with phospholipid analogues indicate that the extent that DnaA inserts into the lipid bilayer is not affected by the presence of *oriC*. However, subtle differences in the region of DnaA that enters the hydrophobic portion of the membrane could not be discerned with this technique (*16*). Future work should help define the involvement of *oriC* as well as identify additional protein factors that participate in the membrane reactivation of DnaA protein.

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