Dissection of the ATPase Active Site of P1 ParA Reveals Multiple Active Forms Essential for Plasmid Partition*

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Background: The ParA partition ATPase drives the dynamic positioning of plasmids in bacteria. Different mutations in a critical lysine of the P1 ParA ATP binding site block different steps in partition. Conclusion: ATP induces multiple transitions in ParA that are necessary for partition. Significance: ParA-like proteins, which transport plasmids and other macromolecular cargo, likely use ATP in similar ways to promote localization.

The segregation, or partition, of bacterial plasmids is driven by the action of plasmid-encoded partition ATPases, which work to position plasmids inside the cell. The most common type of partition ATPase, generally called ParA, is represented by the P1 plasmid ParA protein. ParA interacts with P1 ParB (the site-specific DNA binding protein that recognizes the parS partition site), and interacts with the bacterial chromosome via an ATP-dependent nonspecific DNA binding activity. ParA also regulates expression of the par operon by acting as a transcriptional repressor. ParA requires ATP for multiple steps and in different ways during the partition process. Here, we analyze the properties of mutations in P1 ParA that are altered in a key lysine in the Walker A motif of the ATP binding site. Four different residues at this position (Lys, Glu, Gln, Arg) result in four different phenotypes in vivo. We focus particularly on the arginine substitution (K122R) because it results in a worse-than-null and dominant-negative phenotype called ParPD. We show that ParAK122R binds and hydrolyzes ATP, although the latter activity is reduced compared with wild-type. ParAK122R interacts with ParB, but the consequences of the interaction are damaged. The ability of ParB to stimulate the ATPase activity of ParA in vitro and its repressor activity in vivo is defective. The K122R mutation specifically damages the disassembly of ParA-ParB-DNA partition complexes, which we believe explains the ParPD phenotype in vivo.

The segregation, or partition, of bacterial plasmids typically requires two proteins, a site-specific DNA binding protein that recognizes a DNA partition site, and a partition ATPase or GTPase that is responsible for localization of the plasmid inside the cell (reviewed in Refs. 1–3). Plasmid Par systems can be classified into different types, depending on the sequence of the NTPase involved, which also likely directly reflects their mechanisms of action. The three main classes are characterized by Walker ATPases, actin-like ATPases, or tubulin-like GTPases. The P1 plasmid in Escherichia coli codes for a Walker-type partition ATPase called ParA. Its site-specific DNA binding protein, ParB, binds to the parS partition site. In addition to its direct role in the partition mechanism, P1 ParA acts as a repressor of the par operon by site-specific DNA binding to the par operator, parOP (4, 5). Repression by ParA is strongly stimulated by ParB in vivo and indirectly influences the partition mechanism by regulating parA and parB transcription.

The Walker class of partition ATPases is the most prevalent class that has been identified in plasmid genomes. In addition, parA- and parB-like genes are found in many bacterial genomes, and the ParAs are all of the Walker class (6, 7). ParAs are evolutionarily related to MinD ATPases, which are also dynamic positioning proteins in bacteria (8, 9), and we believe the sequence similarity reflects similarity in their mechanisms of action (3, 10–14). These ATPases form patterns in vivo on a surface (the nucleoid for ParA; the membrane for MinD) in conjunction with a partner protein that stimulates their ATPase activities (ParB for ParA; MinE for MinD). During partition, ParB-parS complexes interact with ParA to promote an uneven distribution of ParA on the nucleoid (15). These and other observations led to the proposal for a diffusion-ratchet model for partition that depends on multiple ATP-specific forms of ParA that are either bound to the nucleoid or free to diffuse in the cell (11). In this model, the interconversion of these forms, which is modulated by ParB, sets up the patterning reaction. In effect, ParB both promotes and follows a wave of ParA on the nucleoid, which results in plasmid movement.

In contrast, actin-like and tubulin-like partition NTPases likely work as their homology to eukaryotic cytoskeletal proteins suggests; they polymerize into self-supporting filaments. Plasmids are attached to the end of the filaments and move apart by insertional polymerization of the NTPase (16–18). Although polymerization of Walker ParAs into filaments has also been proposed (19–21), self-supporting filaments of these ParAs have not been directly observed.

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§ This article contains supplemental Methods, Table S1, Figs. S1 and S2, and additional references.

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ATP binding and hydrolysis play multiple roles in ParA function. ParA possesses a weak ATPase activity that is stimulated by ParB (22), and ATP binding and hydrolysis are necessary for partition (23, 24). P1 ParA has two distinct DNA binding activities, which both require adenine nucleotides but the specificity differs. ADP is a better cofactor than ATP for its site-specific binding activity to parOP (25). ParA also binds to nonspecific DNA (nsDNA), and this activity absolutely requires ATP (11).

In P1 ParA, nsDNA binding requires a slow conformational change in the protein that occurs after binding of ATP, forming ParA-ATP* (11). This change is a key time-delay step that sets up the patterning in response to ParB. The interaction of ParB with ParA and DNA in vitro results in a large protein-DNA complex called NAC, for nucleoid adaptor complex (26). Assembly requires ATP binding and disassembly requires hydrolysis. The complex forms with nsDNA but is stabilized by DNA containing parS. These biochemical properties suggest that NAC represents the transient interactions of plasmids with the nucleoid that are mediated by ParA-ParB interactions and ATP binding and hydrolysis.

We have previously used several versions of P1 ParA that were specifically altered in its three ATP binding site motifs (Walker A, A’, or B) to delineate the roles of ATP binding and hydrolysis (24). The mutations yielded three different in vivo phenotypes. One class behaved as a null mutation; it was defective for both partition and repression. A second was defective for partition but created “super-repressor” alleles of ParA. Their repressor activity alone was equivalent to that of wild-type ParA that had been activated by ParB; that is, they bypassed the requirement for the corepressor activity of ParB. In vitro analyses of these two classes indicated that the null versions were defective for both ATP binding and hydrolysis, whereas the super-repressors could bind but not hydrolyze ATP and were “locked” in the ADP form of ParA (24). In addition, the super-repressor ParAK122Q was unable to form ParA-ATP* or bind nsDNA, consistent with its defect in partition (11). The third class, which has been called ParPD for “propagation-defective,” resulted in plasmid segregation that was worse than that of a null mutation; that is, plasmids were “propagation-defective”, resulted in plasmid segregation that was worse than that of a null mutation. The third class, which has been called ParPD for “propagation-defective,” resulted in plasmid segregation that was worse than that of a null mutation. The third class, which has been called ParPD for “propagation-defective,” resulted in plasmid segregation that was worse than that of a null mutation.

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* Percent of E. coli cells containing the miniP1 plasmid after >15 generations of growth. Experiments were performed at least three times for each sample.

† In this study, we use the mutations in this critical lysine of P1 ParA to further probe the role of ATP binding and hydrolysis in its function. We focus on the ParPD phenotype of ParAK122R to determine its biochemical defect and thus the step in partition that is altered by mutation. We find that ParAK122R is competent for repressor activity, its ATPase activity is damaged, it interacts with ParB but that interaction is not productive, and it is specifically defective in disassembly of NAC partition complexes.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Growth Media—*Escherichia coli* DH5 (recA1)** was used for plasmid maintenance and cloning. *E. coli* DH5 lac::Tc, in which the lacI gene is disrupted by Tn10, was constructed by P1 transduction from E. coli N99 lac::Tc (29) into DH5 that was transiently recA as described previously (30). DH5 lac::Tc (ΔparAB) is a ΔparA ΔparB lysogen (Δ21) phage containing P1 parAB, constructed as described in Ref. 31. DH5Δlac (ΔparOP-lacZ), described previously (24), was the reporter strain for β-galactosidase expression. Briefly, ΔparOP-lacZ contains a translational fusion of lacZ with the start of parA from the par operon, in a Δ21 phage. *E. coli* BL21A DE3(pLysS) was used for protein expression. Plasmids used in this study are listed with relevant genes in Table 1 and described in supplemental Table S1.

Bacterial cells were grown in Luria-Bertani (LB) or M9 media (32). M9gal plates were M9 agar plates supplemented with glucose (0.2%), casamino acids (0.2%), tryptophan (50 μg/ml), and X-Gal (40 μg/ml). Antibiotics were ampicillin (100 μg/ml) and/or chloramphenicol (Cm) (25 μg/ml).

**Proteins and ATPase Assays—**P1 ParB was purified as described previously except that the gel-filtration matrix was Superdex 200 (33). ParA proteins (wild-type and Lys-122 alleles) were purified over two ion-exchange columns as described previously (24, 25) and used for all analyses except ATPase assays. For the latter, wild-type ParA and ParAK122R were further purified over a gel-filtration column to monitor co-elution of ATPase activity with protein. Samples from the monoQ purification step were run on a Superdex 200 column in 50 mM Hepes-KOH, pH 7.5, 500 mM KCl, 0.1 mM Na₂EDTA, 10% glycerol, and 1 mM dithiothreitol.

ATPase activity was measured in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM [γ-32P]ATP (purified as described previously (11)), 100 μg BSA/ml, and 100 μg of son-

### Table 1

**Par⁰⁺ properties of ParAK122R**

<table>
<thead>
<tr>
<th>MiniPl1 plasmid/plasmid encoding parA and/or parB</th>
<th>Par protein(s) present</th>
<th>Stability of miniPl1 in E. coli strain²</th>
</tr>
</thead>
<tbody>
<tr>
<td>plL1 (parS)²</td>
<td>None</td>
<td>&gt;99.5</td>
</tr>
<tr>
<td>pBR322²</td>
<td>None</td>
<td>&gt;99.5</td>
</tr>
<tr>
<td>pEF5</td>
<td>WT</td>
<td>&gt;99.5</td>
</tr>
<tr>
<td>pEF5.8</td>
<td>K122R</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>pEF5.7</td>
<td>K122E</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>pEF5ΔparA</td>
<td>None</td>
<td>&gt;99.5</td>
</tr>
<tr>
<td>pBEF280</td>
<td>None</td>
<td>&gt;99.5</td>
</tr>
<tr>
<td>pBEF281</td>
<td>K122R</td>
<td>60 ± 10</td>
</tr>
</tbody>
</table>

1. The abbreviations used are: nsDNA, non-specific DNA; NAC, nucleoid adaptor complex; LB, Luria-Bertani; Cm, chloramphenicol.

2. DH5 (parB jaggesi) 100 µg/ml, and 100 µg of son-

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**Biochemical Dissection of P1 ParA ATP Binding Site Mutations**

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* Percent of E. coli cells containing the miniP1 plasmid after >15 generations of growth. Experiments were performed at least three times for each sample.

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Plasmid Stability Assays—The miniP1 plasmids pLI1 and pBEF121 confer resistance to Cm and carry the E. coli lacI gene. Experiments were performed in E. coli DH5α lacI - lacZ + cells so that we could use a color screen for plasmid stability. Compatible plasmids expressing ParA and/or ParB (Table 1) were then introduced into the cells with miniP1 by transformation onto LB agar plates containing ampicillin and Cm. Fresh colonies were inoculated into liquid LB with ampicillin, and the culture was grown overnight at 37 °C (~15 generations). Samples were diluted and plated onto M9Xgal plates to yield ~300 to 500 colonies per plate. The phenotypes of these colonies were examined to determine whether the original cells that were plated contained a lacI + miniP1 plasmid. A white colony indicated that the original cell (and most of its descendants) contained miniP1. A blue colony indicated that miniP1 was lost from its cell population sometime before or during the growth of the colony. Blue colonies were patched onto LB plates with Cm to determine whether the original cell contained miniP1. MiniP1 plasmid stability was calculated as follows,

\[
\text{Percent retention} = \frac{\text{white colonies + Cm}^\text{R blue colonies}}{\text{total colonies}} \times 100\% \quad (\text{Eq. 1})
\]

ParA Repressor Assays—The plasmid context used for parA and parB expression was pEF8, in which the genes are expressed from a modified β-lactamase promoter (24). The parB gene was deleted, and the various parA alleles were introduced into pEF8 by cloning (supplemental Table S1). Liquid β-galactosidase assays were performed as described by Miller (34). Briefly, DH5αlac (parOP-lacZ) cells containing a pEF8 plasmid (or pBR322 as the vector) were grown in LB medium with ampicillin at 37 °C until A_600 ~ 0.3. Cultures were chilled on ice, and β-galactosidase activity was measured (34).

RESULTS

The ParPD Phenotype of ParAK122R Is Worse Than Null and Genetically Dominant in Vivo—The ParPD (or propagation-deficient phenotype), was originally characterized with low-copy number miniP1 plasmids that required an active partition system for stability (24, 27). Colonies of E. coli cells carrying these miniP1s could not grow (or grew extremely slowly) compared with those carrying Δpar or par + miniP1 plasmids. The interpretation was that parPD mutations actively destabilized plasmids. We wanted to better quantify the ParPD effect and distinguish it from a lack of stabilizing activity due to simple loss-of-function mutations and thus first developed an alternative assay to measure only destabilization. We constructed a miniP1 plasmid whose stability was independent of Par but was sensitive to ParPD and called pLI1. The copy number of pLI1 was ~8-fold higher than that of wild-type miniP1 due to deletion of the copy control locus incA (35, 36). pLI1 contained parS but not parA or parB. We also measured stability of pBEF121, equivalent to pLI1 except it lacked parS. We next introduced compatible plasmids that contained the parA and/or parB genes under control of a weak constitutive promoter (pEF5 and derivatives (24)) and measured the effect on the stability of pLI1 or pBEF121 (Table 1). First, we confirmed that pLI1 is stable in the absence of Par or in the presence of wild-type parA and parB (>99.5% retention after ~15 generations of cell growth; Table 1). In contrast, parAK122R had a strong destabilizing effect on pLI1 in the presence of parB (18% retention). Removal of parB (pBEF281) or of parS from the miniP1 (pBEF121) eliminated destabilization by parAK122R (Table 1), indicating that the ParA effect is mediated via ParB activity on parS. We also observed that parB alone had a mild but reproducible destabilizing effect (85% retention of pLI1). This observation implies that wild-type parA mitigates the negative effect of parB. Finally, we compared the phenotype of parAK122R with that of the par-defective allele parAK122E. The latter also showed a mild destabilization of miniP1, similar although less severe than that in the absence of parA.

We next asked whether the parPD phenotype was dominant in an E. coli strain that contained wild-type parA and parB inserted into the bacterial chromosome (via an integrated lambda prophage called λparAB) (Table 1). pLI1 stability was not affected by the wild-type ParA and ParB provided by λparAB. In this context, parAK122R with wild-type parB on the compatible plasmid still produced a strong destabilizing effect on pLI1 (13% retention), thus revealing the dominant nature of the K122R allele. When λparAB was the only source of ParB (parAK122R on pBEF281), the destabilization was less strong (60% retention), indicating that the ratio of ParA to ParB does influence the severity of the phenotype. Again, in this assay, parAK122E behaved similarly to deletion of parA.

ParAK122R Possesses Basal Repressor Activity That Is Insensitive to ParB—We previously concluded that the repressor activity of ParAK122R was defective in vivo when compared with that of wild-type ParA (24). However, the nature of the reporter system we used meant that we could not determine whether the mutation damaged the basal repressor activity of ParA or, alternatively, destroyed the ability of ParA to be stimulated by the corepressor ParB (supplemental Fig. S1). To distinguish these possibilities, we used a genetic epistasis approach that took advantage of the super-repressor alleles of parA. These mutations bypass the requirement for the corepressor function of ParB and result in ParAs that are activated in its absence (24). We reasoned that if a mutation such as K122R damaged the ability to be stimulated by ParB, combining it with the super-repressor allele (D251H) would bypass that requirement and result in a fully active repressor. Conversely, if basal activity of ParA was damaged, it would be a weak repressor even in the presence of the super-repressor allele. We examined both ParAK122R and ParAK122E because both were defective for repressor activity in our previous study. Our repressor assay used an E. coli reporter strain with the lacZ gene under control of parOP in the bacterial chromosome and the parA (with or without parB) gene(s) expressed from a constitutive promoter on a plasmid called pEF8 (see “Experimental Procedures” in Ref. 24). We showed previously that, in this assay, wild-type ParA repressor activity alone is very weak, ParB has no repressor activity on its own, and together, ParB strongly stimulates the repressor activity of ParA (24). We repeated the assay and...
compared wild-type ParA with the mutant versions in the absence of ParB (Fig. 1). ParAD251H behaved as a super-repressor, with an activity identical to that of wild-type ParA when activated by ParB. Also, as reported previously, wild-type, K122R, and K122E alleles of ParA repressed very weakly in the absence of ParB. When combined with D251H, however, the two Lys-122 alleles behaved very differently. ParAK122R/D251H behaved identically to ParAD251H, that is, as a super-repressor. Therefore, D251H is epistatic to K122R, indicating that when the requirement for ParB is bypassed, ParAK122R is an active repressor. Conversely, ParAK122E/D251H was a weak repressor, indicating that the K122E change damages the basal activity of ParA.

The basal repressor activity of ParA requires its site-specific DNA binding to parOP, and consistent with the above result, ParAK122E is defective for this activity (24). The epistasis results further predicted that ParAK122R would be proficient at binding parOP in vitro. We purified ParAK122R and compared its site-specific DNA binding activity to that of wild-type ParA. Using both DNase I footprinting (Fig. 2) and gel shift (EMSA) approaches (supplemental Fig. S2), we found that ParAK122R and wild-type ParA bound to parOP with similar affinity. ParAK122R requires ATP or ADP for its site-specific binding to parOP (Fig. 2). ParA with ADP shows more extensive protection of DNA around parOP than is seen with ParA with ATP (Fig. 2). These properties are also seen with wild-type ParA (25). We conclude from both in vivo and in vitro experiments that the K122R change does not damage the intrinsic repressor activity of ParA, but does damage its response/sensitivity to the corepressor activity of ParB. Note that ParAK122R must still be able to physically interact with ParB because the ParPD phenotype is dependent on ParB (Table 1). We further explored the biochemical defects of ParAK122R to determine the step in partition that is defective and is responsible for its ParPD phenotype.

ParAK122R Possesses a Weakened ATPase Activity That Is only Slightly Stimulated by ParB—We next examined the ATPase activity of ParAK122R. Wild-type ParA has a weak ATPase activity that is stimulated by ParB (22). The basal ATPase activity of ParA is quite feeble (turnover 1–2/h), so these assays are generally quite sensitive to small contaminating ATPases during purification. Therefore, to be certain that we could attribute any ATPase activity to ParA, we purified both wild-type and K122R ParAs over one extra purification step, a sizing column, and monitored the ATPase activity across the protein peak that eluted. The rationale was that ATPase activity should follow the peak of the protein if it is due to ParA. In this experiment, ATPase activity was observed to elute from the
column and co-eluted with the ParA peak (Fig. 3, A and B). With these highly purified proteins, both wild-type ParA and ParAK122R showed a weak basal ATPase activity, but the activity of K122R was about one-third of that of wild-type ParA (Fig. 3C). In the presence of ParB, wild-type ParA activity was stimulated ~15-fold. In contrast, the activity of ParAK122R showed very little stimulation by ParB. Therefore, the K122R change has slightly damaged the intrinsic ATPase activity of ParA but strongly damaged its ability to be stimulated by ParB.

**ParAK122R Forms ParA-ATP*—**An important step in the partition process is the ATP-dependent nsDNA binding activity of ParAs (10, 11, 13, 38). An ATP-specific conformational change in P1 ParA is necessary to create the form of ParA that is competent for nsDNA binding, which we call ParA-ATP* and can detect by a decrease in the fluorescence of the single tryptophan residue in ParA. We previously showed that ParAK122E is unable to undergo this change (11), likely because it cannot bind ATP. ParAK122Q (a super-repressor), although able to bind ATP, cannot form ParA-ATP* and is defective for partition. We repeated this experiment with ParAK122R, and, in contrast to the other substitutions at Lys-122, ParAK122R did undergo the ATP-specific conformational change as judged by tryptophan fluorescence (Fig. 4A). This result predicted that ParAK122R would also be able to bind nsDNA. We followed ParA binding to fluorescent Alexa Fluor 514-labeled DNA after addition of ATP or ADP in a stopped-flow experiment. In this assay, DNA binding by ParA quenches the fluorescent signal (11). As predicted, ParAK122R did bind to nsDNA, and this activity required ATP (Fig. 4B). Therefore, we conclude that the partition defect of ParAK122R is not due to a deficiency in its ATP-dependent nsDNA binding activity.

**ParAK122R Is Defective in Disassembly of Nucleoid Adaptor Complexes, NAC—**We next tested the ability of ParAK122R to support the formation of NAC, the large ParA-ParB-DNA com-
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FIGURE 5. ParAK122R, and not K122Q, form large and stable complexes with ParB and DNA. A, sucrose gradient sedimentation analysis of ParA-ParB-DNA complexes. The DNA substrate was the plasmid pBEF165 containing parS (26). In each panel, the lower band represents supercoiled plasmid, and the upper band represents the nicked plasmid form. Reaction mixtures were incubated for 5 min at room temperature, and then separated on a 5 to 20% sucrose gradient over a 60% sucrose shelf, as described previously (26). Fractions were collected from the bottom of the tube, including the sucrose at the very bottom (S), and the DNA was analyzed on agarose gels. Only the region of each gel containing DNA is shown in each panel. The wild-type or mutant ParA and its concentration (in μM), and the presence of ParB, ATP, and/or ATP-S, in each reaction mixture are indicated to the right of each panel. When present, 1.5 μM ParB and 0.5 mM ATP or ATP-S was used. WT, wild-type ParA; KR, ParAK122R; KQ, ParAK122Q. B, complex assembly with ParA proteins as measured by steady-state light scattering. ParB (2 μM) and 0.1 mg/ml of sonicated salmon sperm DNA were preincubated with 1 μM ParA and 1 mM adenine nucleotide (as indicated) for 30 min at room temperature, and changes in light scattering were measured at 467 nm as described previously (26). The version of ParA is indicated below each set of bars. C, ParA-ParB-DNA complexes are stabilized by the K122R mutation in ParA. Reaction mixtures were assembled with ParA (1 μM), ParB (2 μM), and pBEF165 plasmid DNA (50 μg/ml), and preincubated for 30 min at room temperature prior to the addition of ATP (0.5 mM) at t = 0. After a dead time of ~5 s, light scattering was measured in real time. The inset magnifies the first 150 s of the time course. The black line represents ParAK122R, and the gray line represents wild-type ParA. D, complex assembly with ParAK122R requires ParB, DNA, and ATP or ATP-S. ParAK122R (1 μM) and 1 mM adenine nucleotide (as indicated) were preincubated with or without 2 μM ParB and/or 0.1 mg/ml of sonicated salmon sperm DNA/ml for 30 min at room temperature, and light scattering was measured at 467 nm. For B–D, the “light scatter” signal is the change after the addition of nucleotide (light scattering prior to nucleotide addition (20 ± 2 arbitrary units for all samples) was subtracted from the raw data).

plexes that can be detected in sucrose gradient sedimentation and light scattering assays (26). The sedimentation assay measures the change that protein binding has on the migration of DNA through a sucrose gradient. In our previous study, we showed that NAC formation required ParB and ATP. ADP was unable to support complex assembly. The nonhydrolyzable analog ATP-S supported assembly of larger and more stable complexes than those formed with ATP. From these observations, we concluded that NAC assembly and disassembly required ATP binding and hydrolysis, respectively (26).

We repeated these assays with ParAK122R and observed that, in the presence of ATP, ParAK122R was able to support NAC formation (Fig. 5). In sedimentation assays, we used conditions that, with wild-type ParA, would distinguish complexes formed with ATP from those formed with ATP-S (1.5 μM ParA, Fig. 5A) (26). We observed that ParAK122R complexes with ATP were larger than those formed by ParA with ATP, and they resembled those formed by ParA in the presence of ATP-S (Fig. 5A). We then examined NAC formation in light scattering assays and observed a similar result when measured 30 min after addition of nucleotide (Fig. 5B). In addition, as with wild-type ParA, ADP did not promote complex assembly with ParAK122R.

We compared the behavior of ParAK122R to that of the other two Lys-122 alleles, K122Q and K122E. ParAK122Q can bind ATP (24), but it cannot undergo the conformation change necessary to form ParA-ATP* or bind nsDNA (11). This mutant was unable to support NAC in the presence of any nucleotide (Fig. 5A and B). In sedimentation assays, we used concentrations of ParA that generate large complexes (3 μM) to illustrate that ParAK122Q was unable to support any detectable complex formation in the presence of ATP (Fig. 5A). Light scattering confirmed that ParAK122Q was unable to form NAC (Fig. 5B). ParAK122E also could not form NAC (Fig. 5B), likely because the mutation severely damages its ability to bind ATP (24). Together, the results strongly suggest that ParA-ATP* and nsDNA binding activity are necessary to form NAC, and both prerequisites are fulfilled by the K122R allele of ParA.

By monitoring NAC assembly and disassembly in real time, ParAK122R showed a rapid increase in signal after addition of ATP, but the rate of decay was much slower than that of complexes formed with wild-type ParA (Fig. 5C). The latter behav-
ior also resembled that of wild-type ParA in the presence of ATPγS (26). ParAK122R NAC complexes also formed slightly more slowly than those formed by wild type ParA (Fig. 5C, inset). We considered the possibility that the increase in light scatter caused by ParAK122R was due to polymerization or aggregation of ParA with ATP, rather than due to NAC assembly. However, ParAK122R without ParB or DNA showed no light scatter signal with ATP, ADP, or ATPγS (Fig. 5D). As with wild-type ParA (26), complex assembly with ParAK122R absolutely required ParB, DNA, and either ATP or ATPγS (Fig. 5D). The data indicate that ParAK122R can interact with ParB to assemble NAC but is defective in complex disassembly.

**DISCUSSION**

ATP cycling is critical for the dynamics of Walker partition ATPase patterning behavior and partition activity in vivo (15, 23, 24, 38–42). For partition ATPases such as P1 ParA, which act as transcriptional repressors in addition to their partition activities, ATP cycling is also necessary for proper repressor function (24). The phenotypes of specific mutations in the ATP binding sites of these proteins have proven useful probes for the steps in both their partition and repression mechanisms (24, 42). The Lys-122 position in P1 ParA is particularly interesting because four different residues at this position (Lys, Glu, Gln, and Arg) yield dramatically different phenotypes in vivo. Therefore, the ATP binding pocket of ParA is exquisitely sensitive to this position, and these alleles have allowed us to correlate the biochemistry of ParA with specific steps in ParA action in vivo.

*ParAK122R Is an Active Transcriptional Repressor at parOP but Is Insensitive to Activation by ParB*—ParAK122R shows a defect in repression in vivo, and we show that this defect is due to an inability to be activated by ParB. When the need for this activation was bypassed by the D251H allele, repression by ParAK122R was as effective as that by wild-type ParA (Fig. 1). Therefore, the basal repressor activity of P1 ParA is not altered by the K122R mutation, which is also supported by the observation that ParAK122R shows wild-type binding to the par operator parOP (Fig. 2).

Based on the properties of wild-type ParA, we originally proposed that ATP versus ADP binding was a switch between its partition and repressor forms (33). The identification of a second ATP-bound form, ParA-ATP*, and the behavior of ParAK122Q and ParAK122R provides a more detailed biochemical explanation for this switch. We believe it likely that the preferred repressor form of ParA is either ParA-ADP or ParA-ATP but not ParA-ATP*. The conformational change that creates ParA-ATP* reduces site-specific DNA binding activity to parOP and improves nsDNA binding activity. Several observations support this proposal. First, binding of ATP or ADP to ParA both produce a small and rapid increase in tryptophan fluorescence (11), which we have interpreted as dimerization of the protein. As far as this tryptophan can detect, this form is the same for both ATP and ADP. Only the ATP form, however, undergoes the subsequent conformational change to ParA-ATP*. Second, the affinity of ParA for its specific site, parOP, is ~5 to 10-fold higher with ADP than ATP (25), suggesting that the conformational change to ParA-ATP* is responsible for the drop in affinity. Third, the super-repressor ParAK122Q cannot form ParA-ATP* (11), and its affinity for parOP is the same with ATP and ADP (24). It bypasses the activation by ParB because it is locked in the ATP/ADP form. Finally, ParAK122R shows normal basal repressor activity because it forms both ParA-ATP and ParA-ATP*.

*How Are ATP Binding, ParA Conformation, and ATP Hydrolysis Involved in ParA-ParB Interactions during Partition?*—In vitro, the K122R mutation affects several ATP-dependent ParA activities but not all. ParAK122R retains ATPase activity, although reduced (Fig. 3). Its site-specific DNA binding activity to parOP shows similar affinity and nucleotide dependence as that of wild-type ParA (Fig. 2). It can form ParA-ATP*, bind nsDNA, and assemble into NAC, also with the same nucleotide dependence as that of wild-type ParA (Figs. 4 and 5). The activities that are most affected are those that require an interaction with ParB, and the data show that this interaction is altered but not abrogated by the mutation. ParB must interact with ParA because NAC formation requires both ParA and ParB. ParB, however, cannot significantly stimulate the ATPase activity of ParAK122R.

The properties of ParAK122R in NAC assembly and disassembly resemble those of wild-type ParA in the presence of ATPγS (26). Both form NACs that are larger than those promoted by wild type ParA with ATP because the disassembly rate is dramatically reduced. These observations are consistent with the previous conclusion that ATP binding is necessary for NAC assembly and hydrolysis for disassembly (26). The defect with K122R further implicates ParB in complex disassembly. Because ParAK122R can hydrolyze ATP albeit less efficiently than wild-type, it seems likely that the basal ATPase activity of ParA is not sufficient for efficient complex disassembly. This in turn implies that NAC disassembly requires ParB stimulation of ParA ATPase activity.

The inability of ParAK122Q to form ParA-ATP*, bind nsDNA (11), and form NAC (Fig. 5, A and B) provides more information about the requirements for this ParA-ParB-DNA complex assembly. The results argue that the nsDNA binding activity of ParA-ATP* and/or ParA are necessary to form NAC, which is consistent with the ability of ParAK122R to do so. We originally questioned this requirement because ATPγS did not support the conformational change of ParA-ATP to ParA-ATP* (11) but did support NAC formation (26). However, we recently reexamined the effect of ATPγS and observed that it can support the change to ParA-ATP* but only in the presence of DNA (43). Taken together, we believe that ParA-ATP* and nsDNA binding are necessary for partition complexes to interact with nsDNA.

The biochemical steps performed by ParA and ParB that have been identified in the P1 system support a diffusion-ratchet mechanism for plasmid partition (reviewed in Ref. 3). In P1 ParA, nsDNA binding requires the slow conformational change to ParA-ATP* (11). This change is a key time-delay step that is necessary for the patterning reaction. In the absence of ParB, ParA-ATP* will coat the bacterial DNA, whereas ParA-ATP or ParA-ADP is free to diffuse within the cell. Current evidence suggests that ParB-parS partition complexes contain many molecules of ParB bound to the plasmid around parS (31, 44, 45), which creates a high local concentration of ParB.
large ParB complex will locally and persistently stimulate ATP hydrolysis by ParA, resulting in the localized clearing of ParA and the release of the partition complex from the nucleoid. NAC assembly and disassembly in vitro represent the interactions of this ParB-parS complex with the nucleoid in vivo. We have recently recapitulated these steps in a cell-free system and observed them using total internal reflection fluorescence microscopy (43). Using fluorescent proteins and DNA, ParB/plasmid complexes associate with a DNA-carpeted flow cell, disassemble ParA from the partition complex and the surrounding DNA carpet, and then dissociate. The requirements for ATP binding and hydrolysis parallel the biochemistry of NAC assembly and disassembly for both wild-type ParA and ParAK122R. We proposed ParB interaction with the ParA gradient around the partition complex sets up directional movement; in effect, ParB both creates and follows a wave of ParA on the nucleoid.

The similarities among Walker partition ATPases, as well as with MinD, argue that their patterning mechanisms will also be similar (3, 12). There is also a growing list of ParA-like ATPases that are responsible for positioning other macromolecular complexes in bacteria (reviewed in Ref. 3), such as the chemotaxis machinery of Vibrio cholerae (46). We suggest that multiple ATP-dependent conformations will be a property of this class of ATPases and that they will employ similar patterning mechanisms to those of ParA and MinD.

The ParPD Phenotype of ParAK122R Is Due to a Failure to Disassemble Partition Complexes—Quantification of the effects of parAK122R in vivo demonstrates that it actively and severely destabilizes plasmids and that this destabilization is mediated by ParB (Table 1). Our results argue that the biochemical step responsible for the ParPD phenotype is the failure to disassemble NAC and that this failure is because ParB cannot stimulate the ATP hydrolysis activity of ParA. How does the disassembly of NAC in vitro relate to the dynamics of partition complexes in vivo? ParA is likely involved in two types of separation activity. One is the dissociation of plasmids from the bacterial nucleoid as discussed above. The other is separation of plasmid pairs (or groups) so that there are (at least) two plasmids to move apart and segregate to the newly forming daughter cells. These two activities can be, but do not need to be, independent of each other, so the disassembly that we see in vitro could represent both activities. Nevertheless, a failure of one or both would lead to the ParPD phenotype. Plasmids that cannot separate will exist as fewer partitionable units. They will be less stable than when they segregate randomly but individually; that is, without a Par system. Plasmids that are tethered to the nucleoid would also be unable to diffuse and segregate randomly inside cells. Direct visualization of complexes by total internal reflection fluorescence microscopy supports at least the latter possibility (43). Recent examination of a different ParPD allele, ParAD152H, also suggests that there is a defect in separating plasmids (47). A better understanding of the composition of NAC and the interactions required for its assembly will be required to distinguish these different activities in vitro.

ParB Influence on the Conformational States of ParA—ParB is necessary both for the patterning and full repressor activities of ParA. The K122R substitution in ParA modifies, but does not eliminate, the interaction between ParA and ParB. How does ParB affect ParA? We hypothesize that ParB must effectively convert ParA-ATP* to ParA-ADP or ParA-ATP. This would activate the repressor activity of ParA and also strip it from the nucleoid and NAC during the partition reaction. The simplest explanation is via stimulation of ATP hydrolysis. However, we also consider the possibility that ParB can reset the conformation of ParA-ATP* directly to ParA-ATP without requiring hydrolysis. If so, the ParB interaction necessary for this reset may or may not be the same as that necessary for stimulation of ATP hydrolysis. For example, an interaction between DNA-bound ParA-ATP* and ParB may induce a conformational change in ParA that “primes” the ATPase for hydrolysis, but this physical interaction may not be directly coupled to the chemistry. In this way, the chemical step of ATP hydrolysis per se would be uncoupled from the force generation for plasmid motion. ATP hydrolysis would be necessary to reset the conformational state of individual ParA molecules to allow for repeated participation in the plasmid partition process. Such a reset role for ATP hydrolysis occurs with GyrB, the ATPase of DNA gyrase, for example (48). In addition to the multiple conformational changes that take place after ATP binding, it is likely that ParA undergoes additional transitions following ATP hydrolysis, which have time scales that are controlled by ParB and DNA interaction. This biochemical clockwork, coupled with the diffusion properties of the participating molecules at each reaction stage, could control the spatiotemporal dynamics of the diffusion-ratchet system. Further experiments will be necessary to elucidate the rich mechanistic details of the system.

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Biochemical Dissection of P1 ParA ATP Binding Site Mutations

37. Deleted in proof
Dissection of the ATPase Active Site of P1 ParA Reveals Multiple Active Forms Essential for Plasmid Partition
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