RepA protein is the DNA replication initiator of the Pseudomonas plasmid pPS10. RepA dimers bind to an inversely repeated operator sequence in repA promoter, thus repressing its own synthesis, whereas monomers bind to four directly repeated sequences (iterons) to initiate DNA replication. We had proposed previously that RepA is composed of two winged-helix (WH) domains, a structural unit also present in eukaryotic and archaeal initiators. To bind to the whole iteron sequence through both domains, RepA should couple monomerization to a conformational change in the N-terminal WH, which includes a leucine zipper-like sequence motif. We show for the first time that, by itself, binding to iteron DNA in vitro dissociates RepA dimers into monomers and alters RepA conformation, suggesting an allosteric effect. Furthermore, we also show that similar changes in RepA are promoted by mutations that substitute two Leu residues of the putative leucine zipper by Ala, destabilizing the hydrophobic core of the first WH. We propose that this mutant (RepA-2L2A) resembles a transient folding intermediate in the pathway leading to active monomers. These findings, together with the known activation of other Rep-type proteins by chaperones, are relevant to understand the molecular basis of plasmid DNA replication initiation.

DNA replication is a tightly regulated process to guarantee that genetic information is precisely copied once, and only once, each cell cycle (1). This is achieved by means of complex circuits that are distinct in prokaryotes and eukaryotes, although some similarities have become evident recently (2–4). Common essential elements are a cis-acting DNA sequence (the origin of replication) and a trans-acting factor (the initiator). DNA replication initiators are single proteins, or multisubunit complexes, that bind specifically to the origin of replication, where they usually assemble into oligomers. Initiators play two roles: (i) to melt the strands of DNA and (ii) bring to the so-created replication bubble a number of other protein factors. These are required for further extension of the replication fork (helicases), synthesis of an RNA primer (primases), and copying the template (DNA polymerases) (reviewed in Refs. 1 and 2). Any initiator, to be enabled to bind and melt origin DNA, requires an activation step whose molecular basis is a central subject in research on both DNA replication and the cell cycle.

A trait common to initiators is that their function is controlled by ATP binding and hydrolysis (3, 5, 6) defining active or silent conformational states. In the bacterial chromosomal initiator, DnaA (reviewed in Ref. 7), ATP is not strictly required for specific binding to double-stranded origin DNA (oriC). However, it is essential for melting oriC AT-rich repeats, binding to the resulting single-stranded sequences (6) and then for loading DnaB helicase. Subsequent ATP hydrolysis, stimulated by the processivity factor of DNApol III (β-clamp) and other cellular factors, renders DnaA unable to initiate further replication rounds. DnaA can then be reactivated by either acidic phospholipids or DnaK chaperone, which exchange ADP by ATP (7). On their side, Orc1 and Orc5 subunits of the eukaryotic initiator ORC1 also bind ATP, which is required for specific recognition of origin sequences (8). Single-stranded DNA, generated after origin melting, stimulates ATP hydrolysis and exerts a change in the overall shape of ORC (5).

Plasmids are extrachromosomal DNA molecules that borrow from their hosts most of the factors required for replication. However, they often encode their own initiator, termed Rep (reviewed in Refs. 9 and 10). In Gram-negative bacteria, Rep proteins usually bind to directly repeated sequences (iterons) to establish the initiation complex. In addition, some Rep proteins also bind to an inversely repeated sequence (operator) that overlaps with the promoter of the rep genes, thus acting as self-repressors. Dimers of Rep bind to the operator, whereas monomers bind to the iterons (11–17).

RepA is the initiator protein of pPS10, a plasmid isolated from Pseudomonas (18). Mutations in an LZ-like sequence motif, found at the N terminus of RepA (19), enhance dimer dissociation (14). However, a proof for a direct role of LZ in RepA dimerization is still lacking. An helix-turn-helix motif at the protein C terminus is the main determinant of RepA binding to both operator and iteron DNA sequences (20). We have proposed recently (21) that RepA consists of two WH domains (reviewed in Ref. 22). Furthermore, a similar WH fold is found at the C terminus of the eukaryotic/archaeal initiators Orc4/Cdc6 (4, 23), underlining the relevance of studying the molecular mechanism of RepA activation. We had proposed that dissociation of RepA dimers into monomers would result in a structural change altering the relative arrangement and compaction of its two WH domains (21). In the dimers, the C-
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**Experimental Procedures**

**Cloning, Expression, and Purification of RepA-2L2A**—His$_6$-RepA-WT was expressed and purified, and when required, its tag was removed as described previously (21). Mutant repA-2L2A gene was obtained by PCR, using as template the expression vector pRGG-recA-NHis-repA-WT (25) and primers including the following: (i) a SacII site plus sequences coding for the 5’ end of repA but with mutated codons (GCC for Ala-12 and GCG for Ala-19) replacing those for leucines in the putative LZ and (ii) a HindIII site plus a TGA stop codon and sequences complementary to the 3’ end of repA. Mutations were verified by automated DNA sequencing. Expression of His$_6$-RepA-2L2A was carried out at 30 °C in Escherichia coli SGC22097 (clpXP), as performed for RepA-WT (21). His$_6$-RepA-2L2A was purified to homogeneity from inclusion bodies using the same procedure developed for deletion mutants in RepA (21). Protein stocks were kept in 0.5 M (NH$_4$)$_2$SO$_4$, 50 mM NH$_4$-acetate, pH 6.0, 10 mM β-MeEIOH, 0.1 mM EDTA, 10% glycerol. RepA concentrations were calculated based on their absorption at 280 nm in 5.6 mM GuHCl, considering a molar extinction coefficient of 17210 M$^{-1}$ cm$^{-1}$ (www.expsys.ch/tolks/prottparam.html).

**Testing in Pseudomonas aeruginosa**—The Effect of the Histag and the 2L2A Mutation in RepA—pPSEC, a new series of shuttle vectors including the psP10 and ColE1 replicons, was constructed as follows. pRRGB (46) was digested with BamHI to give a 4-kbp fragment including the psP10 replicon (21) and the Kn resistance determinant. This fragment was then filled in with T4 Klenow and ligated to plasmid pET11a (Novagen) to give a 10.8-kbp fragment from pUC18, in which the latter site had also been made blunt-ended. The resulting plasmid (pPSEC1) was then modified to substitute the 5’ half of the repA-WT gene (a 317-bp EcoRI-SphI fragment) by versions encoding a His$_6$ tag (pPSEC2) and the 2L2A mutations (pPSEC3). This was carried out by PCR on the corresponding pRGG-recA-NHis-repA-WT/2L2A templates (see above), using as primers the following: (i) a 41-bp tail with the sequence between the EcoRI site and the ATG initiation codon in repA (18), plus the encoded His$_6$ tag and (ii) sequences from the complementary non-coding strand comprising the SphI site in repA. The three pPSEC constructs were rescued in E. coli JM109, checked by DNA sequencing, and then transformed into P. aeruginosa PAO1024 (18). Relative plasmid copy numbers were determined from cultures of PAO1024/pPSECs in LB medium, supplemented with Kn to 50 μg/ml, at 30 °C. 1.5-mal aliquots were harvested at A$_{600}$ = 0.5, and total lysates were obtained as described (18). 60 μl (1/10) of the lysates were then loaded into 0.8% agarose-Tris acetate EDTA gels, run at 30 V for 24 h, and stained with EtBr. Southern blotting was carried out by transferring the gels to nylon membranes (18) and then hybridized with a 954-bp [α$^{32}$P]dCTP (Amersham Biosciences). X-ray films (AGFA) were exposed and then both the hybridized plasmid bands and the EtBr-stained chromosome were quantified using GelDoc (Bio-Rad). The intracellular RepA levels in P. aeruginosa were assayed by Western blotting, as described (47). 0.5-μl culture aliquots were taken at different A$_{600}$ values. Total lysates were obtained, resolved by SDS-PAGE, and transferred to nitrocellulose membranes by electroblotting. Membranes were incubated with an anti-RepA rabbit polyclonal antiserum (1:1000) and then revealed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:10000) by the ECL procedure (Amersham Biosciences). RepA amount were estimated by comparing the intensities of the specific luminescent bands in pPSEC-containing cells with those in lanes including extracts from pure RepA. The number of cells contributing to the loaded extracts was estimated after serial dilutions of the cultures by counting at a microscope. Viables were then determined by plating on LB-agar supplemented with Kn. Molar concentration of RepA was calculated considering for a P. aeruginosa cell the volume determined previously for E. coli (4.6 × 10$^{-16}$ μl) (48).

**EMSA**—DNA oligonucleotides containing either the operator or the four iteron sequences of psP10 (18) were synthesized and then phosphorylated with T4 polynucleotide kinase. After annealing with their complementary strands, they were cloned into the Smal site of pUC18 and sequenced. Plasmids were cut at the BsaI site and then labeled with 2 units of Klenow and 30 μCi of [α$^{32}$P]dCTP for 30 min at 25 °C. Fragments were excised and purified by electrophoresis in 10% polyacrylamide gels (13). Labeled amounts of plasmid DNA (30 μg of WT or 2L2A) were incubated in ice with radiolabeled DNAs (4000 cpm) in a 20-μl volume of 20 mM Hepes-NH$_4$, pH 7.8, 5 mM β-MeEIOH, 0.1 mM EDTA, 6% glycerol, 50 μg/ml bovine serum albumin, and (NH$_4$)$_2$SO$_4$, provided by the supplied protein, to 0.2 μl. Samples, assembled in ice, were then transferred to room temperature for 30 min before loading into 20% polyacrylamide (29:1)–0.5% TRIS gels. Electrophoresis was run at 150 V and 4 °C. Gels were then dried out and exposed to x-ray films.

**Analytical Ultracentrifugation**—Sedimentation equilibrium experiments were performed in a Beckman XL-A analytical ultracentrifuge. RepA stocks were dialyzed against 0.25 M (NH$_4$)$_2$SO$_4$, 50 mM NH$_4$-acetate, pH 6.0, 0.1 mM EDTA, 0.1-M samples, with different protein concentrations, were displaced into six-channel 0.1-ml cuvettes with a 1.2-cm optical path and centerpieces of epon charcoal. Sedimentation equilibrium gradients were formed at 5 °C by spinning at either 13000 or 15000 rpm. Radial scans were taken at different wavelengths. Base-line offsets were measured at 50000 rpm. Sedimentation velocity of...
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RepA, either alone or complexed with DNA fragments, was performed at 50000 rpm and 5 °C with 350-μl samples displayed into double sector cells. Data from both types of experiments were processed as described (49). The sedimentation coefficient distributions for the RepA-DNA complexes (Fig. 6C, inset) were calculated by direct linear least squares boundary modeling of the sedimentation velocity data (ls-g2(△)) using SEEDFIT (50).

Spectroscopic Assays—Steady state fluorescence spectroscopy was performed in a Fluorlog Johnin-Yvon spectrophotofluorimeter. 350-μl samples of Histag-RepA WT or His6-RepA-2L2A (5 μm) in 0.25 mM (NH4)2SO4, 50 mM NH4-acetate, pH 6.0, 0.1 mM EDTA buffer were displayed into 0.2 × 1.0 cm path length quartz cuvettes and left to equilibrate at 5 °C. Tryp-94 in RepA was then selectively excited (2-nm slit) at 295 nm and emission spectra were acquired between 300 and 450 nm (3-nm slit, Δ = 1:1 nm). For extrinsic fluorescence measurements, bis-ANS (Sigma) was supplied, from a stock in methanol, to a final concentration of 10 μM. Samples were left to equilibrate for 30 min at 5 °C and then were excited at 395 nm. Emission spectra were acquired in the interval of 400 to 600 nm. The contribution of the buffer was subtracted to all spectra. Circular dichroism analysis of His6-RepA WT, Histag-RepA-2L2A, His6-RepA, and RepA-2L2A with DNA was performed in a Jasco-720 spectropolarimeter, using 0.1-cm path length quartz cuvettes. The DNA oligonucleotides tested in the binding assays (RepA sites underlined) were as follows: IIR (operator), G5-AACAAGGACA-GCTCTCCCTGGCCCCCTGTTA-A-3′ (39-mer); IFR (iteron), 5′-ATICGGAGAGAAGGTTTGACAGCCTCTAATGGGAC-TACCC-3′ (45-mer); TEL (unrelated), 5′-GATCCACACCCCCACACCCACACA-CACCCCCACACCCCG-3′ (38-mer); plus their complementary strands. Oligonucleotides were purified, and their concentrations were determined as described (21). Annealing was carried out by slow cooling in Tris-EDTA buffer, and double-stranded DNA stocks (50 μm) were stored at −20 °C. Binding reactions (200 μl) were assembled in ice, in a solution containing 0.145 mM (NH4)2SO4, 50 mM NH4-acetate, pH 6.0, 0.1 mM EDTA, 1 mM β-MeOTOH, 5% glycerol, plus 5 μM protein and/or double-stranded DNA. Spectra were acquired at 5 °C from 320 to 200 nm, using a bandwidth of 1 nm, a 4-s response, 50 nm/min scan speed (0.2-nm steps), and 10 millidegrees of sensitivity. Five to ten spectra were averaged for each spectrum. The spectrum of the buffer was subtracted to all spectra. Circular dichroism analysis of His6-RepA-2L2A to move freely, resulting in an extended conformation of the proposed jack-knife.

RepA-2L2A mutant was constructed by site-directed mutagenesis on an expression vector including repA WT gene fused to His6 (21) (see “Experimental Procedures”). With the aim of testing the effect of the mutations on pPS10 replication in vivo, repA-2L2A was then transferred to a new pPS10-ColE1 shuttle vector (Fig. 2A, pPSEC1), replacing the parental repA WT gene, to get pPSEC3. To check the effect of the His6 tag present in RepA-2L2A, a plasmid in which the repA WT gene includes the sequence coding for the tag was also constructed (pPSEC2). After being rescued in E. coli, plasmids were transformed into P. aeruginosa. The transformation frequency of the repA-2L2A carrying plasmid decreased by three orders of magnitude when compared with those encoding for the WT proteins, whereas no differences were apparent between the tagged and untagged versions of RepA. After four rounds (~80 generations) of replica plating with no selective pressure, the Kn resistance marker had been stably inherited in all clones. However, Southern blot analysis of total lysates from several Kn-resistant pPSEC3 (repA-2L2A) colonies showed that the plasmid was integrated in the chromosome, instead of being replicated autonomously (not shown). This observation suggests that the mutant RepA-2L2A protein is inactive as DNA replication initiator. Growth curves for cells carrying plasmids encoding repA WT show no significant differences between constructs with or without the His6 tag (Fig. 2B), confirming that the fused protein is fully functional as initiator. This was further proved by Southern blotting total lysates from mid-log phase cells, showing that there are no significant differences in plasmid copy number either (Fig. 2C).

Western blot was performed, with anti-RepA polyclonal antiserum, on cells harvested at different stages of growth (Fig. 2B), showing similar protein levels for both versions of RepA WT. Thus, besides being active as initiator, Histag-RepA is also able to regulate its own synthesis (Fig. 2D). Approximated quantification of the RepA amounts yields about 860 protein molecules (or 430 dimers) per cell. This value is around the estimates for the RepA proteins of pSC101 (500 molecules) (41) and RK2 (300 molecules) (52) plasmids but far from the those reported for R6K (about 20-fold higher) (53) and P1 (160 molecules) (47) initiators. It is relevant for the results presented later in this paper to note that pPS10 RepA is found at concentrations over 5 μM across all the growth curves (Fig. 2D).

EMSAs were performed to test whether the failure of RepA-2L2A to act as initiator is due to have its DNA binding prop-


![Image](66x466 to 556x737)

**Fig. 1.** A structural framework for the design of RepA mutants affected in dimerization and conformational activation. A, overview of the WH1 domain of the monomeric RepE54 initiator from mini-F plasmid (Protein Data Bank entry 1REP) (24), with its α-helical elements highlighted in red; α3 and α4 constitute the helix-turn-helix motif, in which the latter is the DNA recognition helix. B, slab view removing the helix-turn-helix to uncover α1 and α2, which include Leu residues conforming to an LZ-like sequence motif (19). The first two of such leucines (Leu-12 and Leu-19), substituted by Ala to generate the RepA-2L2A mutant, are depicted in green. In *cyco* is the single Trp in RepA (Trp-94) that is a crucial part of the network of hydrophobic interactions (distances displayed in white) to which Leu-12 and Leu-19 also contribute. These three residues, absolutely conserved in RepA, RepE54, and other Rep proteins (9, 10, 24), are numbered according to RepA sequence, but the others (in orange) keep the original labeling from RepE54 (24). A dashed purple arrow indicates the expected jack-knife movement that would undergo the N-terminal α1-helix around the hinge including Leu-19, once the hydrophobic network is disrupted by the mutations in RepA-2L2A that, in addition, would hamper any possible dimerization through the leucines.

**Table:** Summary of binding properties altered. Radiolabeled DNA fragments, including either repA operator sequence (Fig. 3A) or the four iterons found at pPS10 origin of replication (Fig. 3B), were incubated with increasing amounts of pure RepA protein, either WT (His6-tagged or untagged) or mutant 2L2A. The latter was with the His6 tag attached, because this increases its solubility and the tag, by itself, does not substantially affect DNA replication in *vivo* (Fig. 2, panels B and C). RepA-WT binding to operator DNA results in a typical pattern of two bands. These correspond to complexes including one or two protein dimers (Fig. 3A, D1 and D2, respectively) (13, 21), to finally yield large protein-DNA aggregates that remain in the well of the gel (W). However, only the first of such complexes, that with a single RepA dimer (D1), is observed with the 2L2A mutant. Its apparent dissociation constant (Kd(app), the protein concentration at which 50% of the DNA probe is bound) is at least 40-fold higher (≥0.7 μM) than that for the His6-tagged RepA-WT (18 nM). Thus, although with a substantially decreased affinity, the RepA protein in which two Leu at its LZ-like motif (Leu-12 and Leu-19) were substituted by Ala seems able to dimerize upon binding to operator DNA. This observation points to the existence of an additional dimerization interface in RepA. It would most probably be the β-sheet in WH1, as proposed for RepE, based in the crystal structure of its monomer (24). Moreover, binding contacts of RepA-2L2A with the operator DNA must be unaffected, because they are made through WH2 (21) that it is intact in the mutant. The affinity of the tagged protein for the operator sequence appears to decrease; about 6-fold more His6-RepA-WT than RepA-WT is required to get a similar amount of bound DNA (Fig. 3A, lanes 1 and 3). In addition, the complex corresponding to the binding of a second RepA dimer (D2) does not appear. Thus, because the *in vivo* data show that both versions (tagged and untagged) of RepA-WT are equally self-regulated (Fig. 2D), the formation of complex D2 seems not essential for repA promoter repression. D1 complexes, established by His6-RepA dimers, show a bit lower electrophoretic mobility than those for the untagged protein, probably because of the extra 4-kDa mass coming from the His6 tag. Concerning the binding of RepA monomers to the iterons at the origin (Fig. 3B) (14), a sharp transition to large protein-DNA complexes that remain in the well of the gel (W), is observed. This occurs even with the minimal amount of untagged RepA-WT tested (7.5 nM), whereas only a tiny fraction of DNA is found in discrete complexes (M1-M4). Although untagged RepA appears to bind cooperatively to iterons, its His6 version does not so much; DNA fragments with one to four iterons bound co-exist (7.5 nM), whereas only a tiny fraction of DNA is found in discrete complexes (M1-M4). Although untagged RepA appears to bind cooperatively to iterons, its His6 version does not so much; DNA fragments with one to four iterons bound co-exist (7.5 nM), whereas only a tiny fraction of DNA is found in discrete complexes (M1-M4). Although untagged RepA appears to bind cooperatively to iterons, its His6 version does not so much; DNA fragments with one to four iterons bound co-exist (7.5 nM), whereas only a tiny fraction of DNA is found in discrete complexes (M1-M4).
determined, we have performed a combined biophysical approach to further characterize the structure of RepA-2L2A.

**Physicochemical Characterization of RepA-2L2A as a Metastable Folding Intermediate**—One of the expected effects of the mutations designed in RepA-2L2A is to interfere with protein dimerization by altering the hydrophobic spine in the putative LZ α-helix (see above) (Fig. 1B). To test the association state of RepA, we have performed sedimentation equilibrium experiments in an analytical ultracentrifuge. We have also addressed whether the N-terminal His6 tag affects RepA-WT dimerization (Fig. 4, panels A and B), before determining the association state of RepA-2L2A (Fig. 4C). In this protein the His6 fusion was kept attached for improving solubility (see above). Ultracentrifugation runs were performed with a RepA range between the minimal concentration giving a reasonable signal to noise ratio with the absorption optics of the ultracentrifuge (1–2 μM) and a maximum close to the limits of RepA solubility (25 μM). RepA-WT remains essentially dimeric through all the concentration range tested (not shown), with a net tendency of the protein to assemble further, but no sign of dissociation. We had reported previously (14) for a fusion with MBP that RepA-WT, in the lowest concentration range tested here, was close to the dissociation equilibrium between dimers and monomers. Now, in light of our new data, that observed dissociation is revealed as a possible steric hindrance effect of the fused MBP moiety, probably caused by its large size (47 kDa). His6-tagged RepA-WT shows a similar behavior to its untagged counterpart, with a slightly more marked tendency to associate beyond dimers (not shown). This is in accordance with data shown above pointing to similar in vivo and in vitro activities for both proteins (see Figs. 2 and 3). Therefore, the rest of experiments described in this paper (see Figs. 5–7) were carried out with the His6-tagged version of RepA-WT, to be compared straightforward with those performed with His6-RepA-2L2A. Sedimentation velocity analysis of His6-tagged and untagged RepA-WT, at either 5 or 15 μM, allowed to determine a sedimentation coefficient (s20,w) for the RepA particle of 4.2 S and a frictional ratio (f/f0) of 1.2 (not shown). These values are again compatible with being the most abundant RepA species dimers, with nearly spherical shape. Thus it is relevant to underline that, based on the present analytical centrifugation analyses and on the conditions used in the spectroscopic studies described below, RepA-WT protein is largely found as stable dimers. Sedimentation equilibrium analysis on RepA-2L2A shows that this mutant protein is polydisperse; at the lower concentrations tested (2–7 μM), a significant monomeric fraction is found (Fig. 4C), together with large aggregates. These appear to become the major species at higher protein concentrations (not shown). Aggregation, described previously (21) for deletion mutants affecting the putative LZ in RepA, can be attributed to the exposure of hydrophobic surfaces (probably an additional dimerization interface; see above) to the solvent. Both the disruption of RepA dimers and the exposure of hydrophobic residues, otherwise buried in the core of the folded N-terminal WH, were expected to arise from the double mutation designed in RepA-2L2A (Fig. 1B).
The possible structural changes induced by the 2L2A mutations were then explored by means of steady state fluorescence spectroscopy. Trp-94, the single tryptophan residue in RepA (18, 21), is a key node in the network attaching Leu-12 and Leu-19 to the hydrophobic core of WH1 (Fig. 1B). Thus, it is expected to be a suitable spectroscopic sensor for any structural modification in its environment. RepA-WT has nine tyrosines (18). Comparing the excitation and emission spectra of RepA with those for free tyrosine and N-acetyl-tryptophanamide solutions we have verified that, at 295 nm, no Tyr residue is excited in RepA. On the contrary, that wavelength falls in the tail of the excitation spectrum of Trp-94 (not shown). Thus, Trp-94, either in RepA-WT or in RepA-2L2A (5 μM), was selectively excited at 295 nm and then fluorescence emission spectra were acquired (Fig. 5A). The maximum emission was achieved at 327 nm (RepA-WT) or 348 nm (RepA-2L2A). The extra band at 335 nm observed in RepA-2L2A emission suggests the presence of two major populations of different rotamers for Trp-94 side chain. The red-shifted emission found for RepA-2L2A is characteristic of exposed Trp residues and it is compatible with the increased solvent accessibility expected for Trp-94 after the release of its hydrophobic linkage to the bipartite α-helix (Fig. 1B). On the contrary, the emission of Trp-94 in RepA-WT, close to 320 nm, is typical of Trp residues buried in a protein core or in a contacting interface (21). The wavelength for the emission maximum in RepA-WT did not change after serial dilutions of the protein sample (tested up to 78 nM; not shown), providing further evidence for its dimeric association state in a broad concentration range. Another clue for a structural change affecting Trp-94 in RepA-2L2A is its enhanced fluorescence emission intensity compared with that for the WT protein (Fig. 5A). To get additional proof for the presence of exposed hydrophobic residues in RepA-2L2A, an extrinsic fluorescence probe was used: bis-ANS, a naphthalene derivative that binds to solvent-accessible hydrophobic patches in proteins, enhancing its fluorescence emission over 450 nm (21). Incubation of bis-ANS (10 μM) with RepA-WT or 2L2A (5 μM) results in spectra with emission maxima around 472 nm (Fig. 5B). The fluorescence intensity at this wavelength is 61% higher for Rep-2L2A than for the WT protein, confirming that the former has a larger hydrophobic surface exposed to the solvent.

As a summary of the results shown so far, RepA-WT is a stable dimer in vitro in a broad (μM) concentration range (Fig. 4A), similar to that found in P. aeruginosa cells (Fig. 2D). In terms of RepA association state (Fig. 4B), DNA binding properties (Fig. 3), or even its activity as a DNA replication initiator in vivo (Fig. 2), there is no significant difference in having a
His$_6$ peptide fused to the RepA N terminus. RepA-2L2A, in which two Leu residues in the putative LZ (Leu-12 and Leu-19) were substituted by Ala (Fig. 1), exposes hydrophobic residues to the solvent, as revealed by fluorescence studies (Fig. 5) and by its enhanced tendency to aggregation. Thus, it exhibits the biophysical properties expected for a metastable monomeric (Fig. 4C) protein folding intermediate (54).

Binding to Iteron DNA Dissociates RepA Dimers into Monomers and Acts as Allosteric Effector on Protein Conformation—

With the aim of deepening our understanding of the structural basis of the recognition of operator and iteron DNA sequences by RepA dimers and monomers, respectively, we have performed a CD spectroscopy study on RepA-DNA complexes (see Figs. 6 and 7). 5 μM His$_6$-tagged RepA-WT protein were incubated with equimolar amounts of double-stranded oligonucleotides, including either the repA operator sequence (1IR, in red), directly repeated iteron (1DR, in blue), and unrelated yeast telomeric (TEL, in green) sequences. Fig. 6A shows overlaid CD spectra of dsDNA oligonucleotides (5 μM, filled symbols) and their complexes with equimolar amounts of His$_6$-tagged RepA-WT (empty symbols); inversely repeated operator (1IR, in red), directly repeated iteron (1DR, in blue), and unrelated yeast telomeric (TEL, in green) sequences.

**Fig. 5.** Steady state fluorescence spectroscopy highlights differences in the folds of His$_6$-tagged RepA-WT and 2L2A mutant. A, fluorescence emission spectra (λexcit = 295 nm) of Trp-94 in RepA-WT and RepA-2L2A (both at 5 μM), whose association states and domain compactness are represented schematically. Dotted vertical lines indicate the emission maxima for the WT (327 nm) and mutant (348 nm) proteins. B, fluorescence emission spectra (λexcit = 395 nm) for the extrinsic fluorophore bis-ANS (10 μM) incubated with either RepA-WT or RepA-2L2A proteins at the same experimental conditions assayed in A. Dashed vertical line marks the emission maximum at 472 nm.

Fig. 6. Iteron DNA as an allosteric effector on both RepA structure and association state. A, near and far UV CD spectra of dsDNA oligonucleotides (5 μM; filled symbols) and their complexes with equimolar amounts of His$_6$-tagged RepA-WT (empty symbols); inversely repeated operator (1IR, in red), directly repeated iteron (1DR, in blue), and unrelated yeast telomeric (TEL, in green) sequences. B, far UV CD spectra of His$_6$-RepA-WT and 2L2A proteins at 5 μM (circles), plotted together with the result (diamonds) of subtracting the spectra of naked DNAs to the protein-DNA complexes. C, HPLC gel filtration elution profiles of duplicates of the samples in A, maintaining the same color and symbol codes. Drawings close to the peaks interpret the different DNA fragments and their complexes with His$_6$-RepA-WT. Inset shows the sedimentation coefficient distributions calculated for both protein-DNA complexes, which were spun down in an analytical ultracentrifuge just after peak elution.
at about 208 and 222 nm, a signature for a significant proportion of α-helical secondary structure (33%), are one type. These correspond to free Rep-A WT and in complex with the operator oligonucleotide 1IR. (ii) Curves with a broader minimum around 215 nm, attributable to an increase in the β-sheet component (by about 4%) at the expense of former α-helices, as observed for RepA WT in complex with the iteron oligonucleotide 1DR, are another type. It is relevant that RepA WT does not change its spectrum and thus its structure to a detectable extent, when it is free in solution or bound to the operator. This is coincident with the fact that RepA is essentially an homogeneous dimer in solution at this concentration (Fig. 4B) and that it binds to that DNA sequence as a dimer (Fig. 3A) (14). However, when it binds to the iteron DNA as a monomer (Fig. 3B) (14) its spectrum resembles that of the mostly monomeric RepA-2L2A mutant (Fig. 6B). We have failed to acquire clean spectra for complexes between this mutant protein and any DNA, because a cloudy precipitate appears. Although less severe, protein aggregation is also observed, in the form of noisy spectra, for the nonspecific complexes between RepA WT and DNAs of unrelated sequence, such as TEL (Fig. 6), other mixed sequence oligonucleotides, and poly(dI-dC) (data not shown).

As a summary, our spectroscopic studies show that RepA has a distinct secondary structure composition when bound to the inversely repeated operator sequence or to the directly repeated iteron DNA.

To correlate the observed conformational change with the association state of RepA, we carried out EMSAs (not shown), size exclusion chromatography (Fig. 6C), and sedimentation velocity (Fig. 6C, inset) on the same samples tested by CD. EMSAs consistently result in that the RepA-operator complex slightly has lower electrophoretic mobility than the RepA-iteron band (not shown). Taking into account that the free iteron oligonucleotide (1DR) is 6 bp larger than the operator one (1IR), this result is compatible with having a larger protein mass in the latter complex (a dimer) than in the former (a monomer). However, EMSAs are not conclusive, because the minute difference in the electrophoretic migration between both types of complexes could be also because of disparate bending behavior of the bound DNA fragments. For the sample including RepA and the unrelated oligonucleotide TEL, no discrete retarded band is visible but some smearing, indicative of low affinity, nonspecific binding (not shown). When the samples were injected into a size exclusion column the specific RepA-oligonucleotide complexes remain stable, and their hydrodynamic behavior (Fig. 6C) was unambiguous. The relative elution position of the peaks corresponding to bound DNAs is the reverse of that for the unbound species (the assay is sensitive enough to detect the aforementioned slight difference in the sizes of free oligonucleotides). This fact can be explained if the protein mass associated with the smaller operator DNA piece (1IR) is substantially larger (a dimer) than that associated with the longer iteron (1DR) fragment (a monomer). The possibility of having just one subunit in a RepA dimer bound to the iteron, as proposed for the Rep protein of R6K plasmid (16), is thus very unlikely. Such a complex would be expected to elute just before the RepA-operator one. To characterize further the nature of the complexes eluted from the gel filtration column, analytical ultracentrifugation was performed. Unfortunately, RepA-DNA complexes dissociate during the time course required to achieve sedimentation equilibrium (not shown). However, the interpretation proposed above is in agreement with the results of sedimentation velocity experiments performed, immediately after the gel filtration runs, with the peak fractions. There is a substantial difference in the sedimentation coefficients for the RepA-1IR (6.5 ± 0.2 s) and the RepA-1DR (3.8 ± 0.2 s) complexes. These sedimentation coefficients are only compatible with being the RepA-1IR complex significantly more compact (βf₀ = 1.2) than RepA-1DR (βf₀ = 2.0). The latter value fits with the elongated shape of a complex between a RepA monomer and an oligonucleotide with identical length to 1DR, modeled on the crystal structure of Rep54 (24) (not shown). Therefore, our results clearly show that the structural changes in RepA, coupled with binding to iteron DNA (Fig. 6B), are linked to dissociation of the otherwise stable protein dimers (Fig. 4B) into monomers (Fig. 6C).

It is noteworthy that, for unbound RepA, we have not found any sign of dissociation of dimers after incubation times ranging from 30 min (spectroscopy; see Figs. 5 and 6) to 16 h (sedimentation equilibrium; see Fig. 4). On the contrary, upon incubation with origin DNA RepA dissociation (Fig. 6C) and the coupled structural transformation (Fig. 6D) occur within a few minutes. Because serial dilutions of unbound RepA exhibit the fluorescence emission spectrum characteristic of dimers (Fig. 5A) at least up to low nanomolar concentrations (not shown), the possibility that at 5 μM as a small fraction of monomeric RepA, in equilibrium with dimers, would bind to the iteron sequence and that this event would drive monomer formation seems unlikely. In summary, there are solid basis to affirm the following. (i) Because the Kₐ for RepA dimers ap-

![Graph](image-url)
Binding to Iteron DNA Changes RepA Structure

Pears to be in the low nanomolar-subnanomolar range, in our experimental (micromolar) conditions the equilibrium is well displaced toward the dimeric species. (ii) Along broad time courses no monomeric unbound fraction has been detected for RepA. Thus the reported induction of RepA dissociation by iteron DNA seems to be genuine.

Thermal denaturation analyses performed by optical techniques, such as CD spectroscopy, provide valuable information on the thermodynamic stability of proteins (4, 21) and their complexes with DNA (55). We have carried out this kind of approach to study the effect on RepA stability of binding to the DNA sequences described above (Fig. 6). Inspection of the CD spectra in Fig. 6A reveals that, at 228 nm, the contribution of the oligonucleotides to the ellipticity (θ) of their complexes with RepA is essentially null, whereas that from the α-helical and β-sheet structural elements in RepA (Fig. 6B) is significant. Thus, we plotted the variation of Δ228 with temperature for each kind of RepA-DNA complex (Fig. 7) and then compared the resulting curves. Because thermal denaturation of RepA and its complexes is irreversible, a detailed thermodynamic analysis of the CD profiles was precluded. The temperature at which 50% of protein molecules unfold (Tm) was determined, because it is essentially unaffected by irreversibility and gives an idea on protein stability. Binding to operator DNA (1IR) stabilizes RepA, relative to its unbound state, by 12.4 °C, whereas binding to iteron DNA has a smaller effect (4.8 °C). The more pronounced slope of the curve for 1IR complexes indicates that RepA denaturation becomes more cooperative upon operator binding by the dimers. This effect is also observed for the complexes of RepA monomers with iteron DNA (1DR), although to a lesser extent. However, binding to non-specific sequences (TEL) severely decreases the stability of RepA (by 11.5 °C), approaching the Tm values measured for the mostly monomeric folding intermediate RepA-2L2A (Fig. 7) and for an N-terminal partial deletion derivative (ΔN37) reported previously (21). This observation suggests that, as described for some restriction endonucleases (56), nonspecific DNA could, to some extent, exert conformational changes in RepA (Fig. 6B) enabling it to scan for specific iteron sequences. However, the resulting RepA molecules seem to be unstable folding intermediates that, because of the exposure of hydrophobic residues prone to aggregation (see above), would recruit molecular chaperones. Therefore, iteron DNA not only acts as allosteric conformational effector on RepA dimers (Fig. 6B) but also has a role in stabilizing the structure of the resulting monomers.

**DISCUSSION**

**RepA-2L2A: a Folding Intermediate in the Pathway from Repressor Dimers to Initiator Monomers**—In this paper we have described the design of RepA-2L2A, a mutant in the protein that initiates DNA replication of the *Pseudomonas* plasmid pPS10, based on the available three-dimensional structure of the monomer of a homologous initiator (24). RepA-2L2A carries a double Leu → Ala substitution (Leu-12 and Leu-19) in the LZ-like motif found in the protein (14, 19, 21). These mutations were expected to enhance dissociation of an otherwise dimeric protein into monomers and to destabilize the hydrophobic core of the latter (Fig. 1). Functional characterization of RepA-2L2A indicates that it is inactive as initiator of replication (Fig. 2). RepA-2L2A has affected its binding to iteron DNA sequences (Fig. 3B) and reduced its affinity for operator DNA (Fig. 3A), where RepA binds as a dimer (13, 21). Hydrodynamic studies (Fig. 4) show that RepA-2L2A can be largely found as a monomer in a narrow micromolar range, forming nonspecific aggregates at higher concentrations. Dimerization of WH domains (23) through hydrophobic residues in the α-helices equivalent to α2 and α4 in Rep proteins (Fig. 1) has been described for the complex between the eukaryotic transcription factors E2F4 and DP2 (57). However, a role in dimerization for the antiparallel β-sheet found at the N-terminal WH domain of RepE54 is also possible (24). To determine whether the LZ-like motif has a direct contribution to the interprotomeromic contacts in RepA dimers, or whether it rather favors RepA association indirectly (e.g., stabilizing the dimeric conformation), will require solving the three-dimensional structure of a Rep protein dimer. Spectroscopic evidence (Fig. 5) supports that RepA-2L2A resembles a transient folding intermediate, with hydrophobic residues partially exposed to the solvent, expected to occur in the pathway from repressor dimers to initiator monomers. This is the first report on the isolation and characterization of a conformational intermediate in a Rep-type initiator protein. In addition, we have shown that short His6 fusions to RepA have a minor effect on the association state of the protein in vitro (Fig. 4B), which remains as a dimer in a broad micromolar range. The His6 tag does not alter RepA initiator activity in vivo (Fig. 2), despite showing some reduction in its binding cooperativity to iteron sequences in vitro (Fig. 3B).

**The Allosteric Effect of Iteron Binding on RepA Conformation and Association State**—At the same concentration in which RepA is a dimer (Fig. 4) with no sign of dissociation into monomers, binding to an oligonucleotide encoding for a single iteron origin sequence results in dissociation of RepA into monomers (Fig. 6C) and a change in protein secondary structure, which becomes similar to that for the monomeric intermediate RepA-2L2A (Fig. 6B). On the contrary, the structure of RepA dimers appears to be unaltered by binding to the inversely repeated operator sequence (Fig. 6B). Interestingly, binding to both types of DNA stabilizes RepA against thermal denaturation, although to a different extent (Fig. 7). This fact also points to differences in the structures of RepA dimers and monomers. In the same experimental conditions, nonspecific DNA oligonucleotides result in precipitation of RepA and in destabilization of the structure of the protein (Fig. 7). Structural changes in a number of DNA-binding proteins upon specific ligand recognition have been previously reported (44, 45, 56) as cases of allosterism. However, apart from being found in some viral initiators (58, 59) and in ORC with single-stranded DNA (5), they are new events in the initiation of DNA replication in bacterial plasmids.

The precise mechanism through which iteron DNA exerts the allosteric effect on RepA structure described in this paper remains to be determined in its molecular details. It is worth noting that in the eukaryotic transcription factor Ets1 an α-helix (α1-2) is found at the N terminus of the three α-helices of its WH domain (60), packing against the first helix very much as α1 and α2 do in Rep proteins (Fig. 1B). A phosphate in DNA backbone triggers an allosteric transition in Ets1 α2-1 by establishing a hydrogen bond with the amide NH of a Leu residue at α1 N terminus. This corresponds in RepE54 monomers with Arg-33 (Fig. 1B), which also binds to iteron phosphate backbone (24). Arg-33 in RepE54 consistently aligns with hydrophobic residues (mainly Leu, as in Ets1) in RepA, other members of its family (9, 10, 24), and the eukaryotic and archaeal initiators Orc4/Cdc6 (4). Thus a way is opened to a possible general role of this residue in triggering allosteric transitions in WH initiator proteins.

**The Role of Chaperones and Specific Origin Sequences in the Structural Activation of Rep Proteins**—At the concentrations it is found in *Pseudomonas* cells in vivo (Fig. 2D), RepA is dimeric
(Fig. 4), acting as transcriptional repressor (13, 20). It has been found for P1 (34, 42) and pSC101 (41) plasmids that their Rep proteins can spontaneously dissociate into monomers just by dilution to low/sub-micromolar concentration. However, besides pPS10 RepA, the initiators of F (12), R6K (16, 61), and RK2 (15, 36) plasmids have their \( K_d \) values in the low nanomolar range. For the latter the requirement for an active mechanism in Rep dissociation, and in the coupled conformational change, thus remains as a bottleneck. Our findings on the ability of an iteron sequence to induce structural transitions in RepA confer a property to origin DNA that was attributed previously to chaperone action alone (reviewed in Ref. 30).

Based on data presented in this paper, we propose that the structural changes in Rep that are coupled to dimer dissociation would imply the following: (i) releasing protein-protein interactions between protomers, involving the LZ-like motif and/or the \( \beta \)-sheet in the first domain (21, 24); (ii) remodeling the hydrophobic core of WH1, where the relevant Leu residues (Leu-12 and Leu-19) are tightly packed with Trp-94 (Fig. 1); (iii) an increase in \( \beta \)-strand structure at the expense of the \( \alpha \)-helical components (Fig. 6B). The rearrangement experienced by Rep would necessarily generate transient intermediates with properties similar to those of the RepA-2L2A mutant, namely having hydrophobic sequence patches exposed to the solvent and a loosely folded core (Fig. 5), both features of molten globules (54). This explains why monomeric RepA, when free in solution and in the absence of iteron DNA, is prone to aggregation through WH1 (see above) (21). To cope with these challenges to Rep activation, different, albeit complementary, molecular mechanisms can be envisaged.

Chaperones can directly dissociate Rep dimers and simultaneously change the conformation of the monomers, to make them competent for iteron binding. This has been reported for the DnaK-DnaJ-GrpE triad (11, 31, 33), ClpA (32, 43), and ClpX (36). These could target WH1 in Rep; ClpA recognizes a region at the N terminus of P1 Rep (residues 10–70) (62) as the first step toward unfolding, either for its activation as initiator or for degradation by the associated ClpP protease (37, 38). DnaK, on its side, recognizes a hydrophobic patch (residues 36–49) in the same Rep protein (63), which in pPS10 RepA corresponds to a sequence (residues 91–105) comprising the Trp-94 residue discussed above. We had shown previously (64) that pPS10 replication in vitro is sensitive to DnaK levels. Although they physically interact (4), if DnaK is acting on pPS10 RepA dissociation remains to be determined. For those Rep dimers that dissociate spontaneously, chaperones could bind to exposed hydrophobic regions (65) in the monomeric intermediates, thus protecting them from falling into local energy minima that would act as kinetic traps in the pathway leading to Rep folding and iteron binding. Otherwise, large Rep aggregates could arise from the accumulation of monomeric intermediates, but it seems feasible that the combined action of several chaperones, such as ClpB plus DnaK-DnaJ-GrpE (66), could rejuvenate them into active initiators.

The allosteric conformational changes induced in RepA by iteron DNA (Fig. 6) would constitute another means to get the monomeric species, specially for those Rep proteins that are stable dimers at the concentrations they are found in vivo (Fig. 2D) (16, 52, 53). This way could be favored by the cooperativity of Rep binding to DNA (Fig. 3B). Alternatively, iteron DNA could also capture and stabilize the monomeric folding intermediates produced after spontaneous dissociation of Rep dimers, as proposed above for chaperones. An implication of this new activation mechanism is that only a fraction of the total amount of Rep molecules, coincident with the number of iteron sequences, would be eventually activated whereas the others would remain dimeric. On the contrary, there is no obvious means to limit the number of active Rep monomers generated by the chaperone-mediated route that could be accumulated in excess over the levels required for regulated initiation. In addition, the allosteric binding of iteron DNA to RepA implies that the structural changes associated to monomerization are intrinsically accessible to Rep proteins and not necessarily the exclusive product of chaperone action.

In plasmids containing iteron sequences at their origins of replication, the most favored model for negative control of initiation implies pairing distant Rep-bound iteron repeats (“handcuffing”) (30, 67). Except in R6K plasmid (16) handcuffing appears to be mediated by Rep monomers. However, in some Rep proteins, a number of mutants have been isolated that, being monomeric hyperactive initiators, fail to pair iterons (40, 68). Most of such mutations fall in a putative additional dimerization interface in Rep that, besides the LZ-like motif, we have discussed for RepA-2L2A. Thus, Rep proteins activated by the allosteric route resemble the behavior of those hyperactive mutants. The minimal requirements for pairing are two DNA fragments containing each a single iteron repeat (69), but we have failed to detect handcuffing even at 5 \( \mu \)M RepA-iteron complex concentration (Fig. 6C). Our model leaves open the possibility for the existence of two kinds of Rep monomers, correlated with their opposite functions as initiators or negative regulators of plasmid DNA replication. Thus, unwinding of origin DNA after the formation of the Rep-iteron nucleoprotein complex might induce a further conformational change in the monomers, as described for the chromosomal initiators ORC in eukaryotes (5) and DnaA in bacteria (6). RepA would then become competent for origin pairing but disabled for a new round of initiation on recently replicated DNA.

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