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Cooperative binding mode of the inhibitors of R6K replication, π dimers

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Abstract

The replication initiator protein, π, plays an essential role in the initiation of replication of plasmid R6K. Both monomers and dimers of π bind to iterons in the γ origin of plasmid R6K, yet monomers facilitate open complex formation while dimers, the predominant form in the cell, do not. Consequently, π monomers activate replication while π dimers inhibit replication. Recently, it was shown that the monomeric form of π binds multiple tandem iterons in a strongly cooperative fashion, which might explain how monomers out-compete dimers for replication initiation when plasmid copy number and π supply are low. Here, we examine cooperative binding of π dimers and explore the role these interactions may have in the inactivation of γ origin. To examine π dimer/iteron interactions in the absence of competing π monomer/iteron interactions using wild-type π, constructs were made with key base changes to each iteron that eliminate π monomer binding yet have no impact on π dimer binding. Our results indicate that in the absence of π monomers, π dimers bind with greater cooperativity to alternate iterons than adjacent iterons, thus preferentially leaving intervening iterons unbound and the origin unsaturated. We discuss new insights into plasmid replication control by π dimers.

It is believed that all naturally occurring plasmids employ efficient copy-control mechanisms to ensure their maintenance at a reasonably constant copy number from cell to cell. The antibiotic resistance plasmid, R6K, is maintained at a steady 15–20 copies per chromosome[1] in a wide variety of bacterial hosts.[2] For this to occur, regulatory controls at the step of replication initiation work to increase low plasmid copy numbers and reduce elevated ones.[3]

Controlled replication of plasmid R6K requires two plasmid-encoded elements: the iterons in the γ origin of replication (γ *ori*) and the *pir* gene that encodes the replication (Rep) protein, π (Figure 1)[4–8] γ *ori* activation requires the binding of monomers of π protein to the seven 22 base pair (bp) iterons within γ *ori* that are adjacent to an A+T-rich region of the plasmid.[9–11] The binding of π monomers to iterons causes an apparent bending of the origin DNA, allowing the nearby A+T-rich region to melt, the replication complex to bind, and replication to start uni-directionally from a specific site within the A+T-rich region.[11–13]

While monomers of π activate replication, dimers of π appear to inhibit replication through several different mechanisms (Figure 1).[9,14–17] π dimers bind a non-iteron site within the A +T-rich region in proximity to the start sites for leading strand synthesis.[18] It has been hypothesized that π dimers negatively modulate the priming step of the replication process by

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binding to this site.[18–20] π dimers also inactivate γ *ori* by binding iterons. π was the first of its family of Rep proteins that was shown to be capable of binding iterons as a dimer.[9] Since then, two more Reps were also shown or inferred to bind iterons as dimers, thus establishing a new trend in Rep/iteron control.[21–23] Although a π dimer can bend iteron DNA to the same extent as a π monomer,[12] π dimers cannot induce DNA strand separation,[16] yet the binding of π dimers to iterons has several consequences for γ *ori* replication control.[9,15–17]

A π monomer contacts both the 5' half and the 3' half of the iteron through the C- terminal winged-helix (WH2) and the N-terminal winged helix (WH1), respectively, but a π dimer only contacts the 5' half of each iteron, including a highly conserved TGAGnG motif, with the WH2 of one of its subunits.[24–27] This potentially allows the WH2 motif of the second subunit of the dimer to contact another iteron-bearing sequence,[9,26] for instance, either of the other two functional *oris* of R6K, the α iteron or the β half-iteron, which are the active *oris in vivo*.[28,29] Such 'looping' is believed to transmit the replication signal from the internal iteron cluster at γ *ori* to the distant *oris*.[30] The second subunit of the dimer may also participate in handcuffing, which is the coupling of two *oris* by Rep-Rep interactions, blocking the initiation of replication for each plasmid involved.[31] Finally, dimers may inhibit over-replication by simply prohibiting monomers from binding to a sufficient number of iterons for activation (this number might be as little as 5),[32] preventing the *ori* from achieving the bent conformation required for open complex formation.

Because π is not limiting in the cell and both π monomers and dimers bind to iterons, there is likely to be a continuing competition for π binding sites. Therefore, a mechanism must be employed by which monomers out-compete dimers for iteron binding and *ori* activation when plasmid copy number is low while dimers out-compete monomers to prevent over-replication when plasmid copy number is high.

A partial explanation was provided recently when it was shown that monomers of π bind cooperatively to two adjacent iterons.[33] It was also shown that a dimer-biased variant of π, π•M36A^M38A binds independently to two adjacent iterons.[33] However, these studies did not reveal how dimers bind to more than two iterons, or if trends are the same for adjacent and alternate iterons. Also, it remains unclear whether the binding properties of π•M36A^M38A are similar to π•wt. This work attempts to answer these questions and demonstrates that dimers of π•wt bind poorly to nearest neighbor iterons but exhibit strong cooperative binding to alternate iterons. These findings cast a new light on how replication control of this complex replicon can be achieved.

Mutations in the "right" side of the iteron prevent π monomer binding but not π dimer binding

To accurately quantify the binding of dimers of π•wt, it was necessary to first eliminate binding of the monomeric form. Since both monomers and dimers bind to iterons, and preparations of π•wt contain both monomers and dimers, in order to use π•wt in these experiments, iterons had to be mutated in a way that prevented monomers from binding yet had no impact on dimer binding. Our lab previously demonstrated through contact probing with a single iteron that monomers of π contact bases throughout the 22 bp iteron while dimers only contact the "left-half" of the iteron.[26] Furthermore, it was previously reported that collective mutations in the 14th, 17th, and 18th bp of a single iteron prevented monomers from binding both *in vitro* and *in vivo*.[26] However, these mutations were tested in one isolated iteron and were not tested with two or more iterons in tandem. Thus, EMSA was carried out with π•wt and labeled DNA probes containing either three tandem wt iterons or three tandem iterons with mutations in the 14th, 17th, and 18th bp (Figure 2). To distinguish complexes containing monomers from complexes

containing dimers, π•M36A^M38A was used as a size control because this variant binds iterons predominantly as a dimer.[16,25]

Results in Figure 2 show that mutations in the 14th, 17th, and 18th bp of each iteron, collectively, eliminate π monomer binding to three tandem iterons *in vitro*. This is true for π•wt and a monomer-biased variant, π•P106L^F107S. Conversely, πdimers bind equally well to wt iterons and iterons with mutations in the 14th, 17th, and 18th positions (the "monomer-only" side) of the iteron *in vitro*.

Quantification of cooperative π dimer binding to adjacent and alternate iterons

The above monomer-deficient iterons with mutations in the 14th, 17th, and 18th bp were used to quantify the cooperativity of π dimer binding to two adjacent iterons and two iterons separated by 22 bp or 44 bp (one or two iterons, respectively). Because changes in the sequence of the iterons might change the intrinsic architecture of the DNA, which could affect binding of π, a minimal number of base changes were made to the intervening iterons to prevent dimer binding. It was shown previously that mutating the 7th and 9th bp of the iteron greatly reduces π dimer binding (and π monomer binding as well).[26,34] Iterons with mutations at the 14th, 17th, and 18th bp (the monomer-only side) that are still proficient for dimer binding, are represented as (_…). Additionally, iterons with mutations in the 7th and 9th bp as well as the 14th, 17th, and 18th bp are incapable of binding dimers and monomers and are represented here as (X…). Thus, three constructs of equal length were created with two dimer-binding-proficient iterons adjacent to each other (_…|_…|X…|X…), separated by 22 bp (_…|X…|_…|X…) and separated by 44 bp (_…|X…|X…|_…).

Cooperativity is expressed as the value k_{12}, a constant obtained from binding equations derived by the statistical mechanical approach,[35] and fit to data from titrations of protein with DNA. Values of $k_{12}>1$ represent positive cooperativity. Figure 3(a–c) shows a representative gel shift titration of π • wt with each of the above probes. The fraction of total DNA that was free, bound to one dimer, and bound to two dimers was quantified and plotted as a function of π concentration (Figure 3(d–f)). To calculate the cooperativity coefficient, k_{12}, data in Figure 3 (d–f) were subjected to a least-squares linear regression analysis using equation (1c). This analysis provided the macroscopic binding constants, K_1 and K_2, which were used to calculate k_{12} using equation (2), as described previously.[33] The values for K_1 and K_2 are displayed in Supplementary Table 2.

It was found that dimers of π•□ □ bind poorly to nearest-neighbor iterons (_…|_…|X…|X…) (k_{12} = 11.5±9.6) but in contrast, they bind with significant cooperativity to alternate iterons (_…|X…|_…|X…) (k_{12} = 92.2±7.3). Furthermore, it was found that π dimers bind with similar cooperativity to iterons separated by 22 and 44 bp (_…|X…|_…|X…) and (_…|X…|X…|_…) (k_{12}=97.7±3.3). Thus, these data demonstrate that the cooperative binding mode of π dimers is dependent on iteron spacing.

Discussion

A major complication in the study of mechanisms that regulate γ *ori* activation is the intriguing quality of π that monomers and dimers bind to overlapping sequences of DNA.[26] This property of π has made it difficult to examine the binding of one form in the absence of the other. Many π variants that are biased toward monomer or dimer binding have been used to circumvent this complication.[16,17,25,36] However, an accurate assessment of the interaction of iteron DNA with strictly monomers or dimers of π•wt as been difficult to achieve because it has not been possible to eliminate πdimers from binding iterons without also eliminating π monomer

binding. However, this work shows that it is possible to do the reverse and use key iteron mutations in multiple tandem iterons to completely eliminate π monomer binding while leaving π dimer binding unaffected. This has allowed us to calculate the binding cooperativity of π dimers without monomer interference.

The current study was based on our previous observation that monomers of π•wt bind iterons cooperatively *in vivo* and monomers of the monomer-biased variant π•P106L^F107S bind cooperatively *in vitro*.[33] With this study, we have demonstrated that dimers of π•wt bind with greater cooperativity to alternate iterons than adjacent iterons. The magnitude of this cooperativity (k_{12}≈100) is less than that of two π monomers binding to tandem iterons (k_{12}≈210).[33] Thus, the hierarchy of π/iteron cooperativity *in vitro* appears to be as follows: monomers bind with the highest cooperativity to adjacent iterons, then dimers bind with moderate cooperativity to alternate iterons and dimers bind with very low cooperativity to adjacent iterons.

These results fit into a working model for the regulation of replication initiation by the disparate cooperative binding properties of monomers and dimers of π(Figure (6)). In this model, at low concentrations of intracellular π•□□, the monomeric form of π binds with strong cooperativity to a sufficient number of iterons to activate γ *ori* and initiate replication.[33] Likewise, at slightly elevated levels of π, host factors such as Integration Host Factor (IHF) and DnaA bind to neighboring sites in γ *ori* and may work to inhibit binding of dimers to iterons,[13,37] thus allowing monomers with stronger cooperative binding to continue to bind and activate γ *ori*. This fits with previous data showing that IHF and DnaA are only needed at moderate and high levels of π protein *in vivo*.[13,37] However, as the concentration of π increases, dimers become the predominant form of π in the cell, outnumbering monomers, and perhaps override the relief of inhibition provided by IHF and other host factors. At this level of π, dimers bind preferentially to alternate iterons of γ *ori*, resulting in incomplete saturation of the *ori*. Hypothetically, incomplete saturation would explain why dimers do not induce DNA strand separation even though they can bind to an iteron and bend DNA to a similar degree as do monomers. When dimers bind to alternate iterons, they are insufficient to facilitate the localized bending of γ *ori* iteron DNA necessary to induce strand separation of the nearby A+T rich segment of DNA. They may also initiate looping to shuttle the replication complex to the α and/or β origins of the same plasmid.[30] At the highest concentrations of π, which occur at the highest plasmid copy number, dimers may engage in origin pairing (handcuffing) with other origin-bound π dimers, thus negatively regulating replication initiation by yet another mechanism.[31] Thus, R6K has evolved multiple mechanisms to control its replication initiation, and the hierarchical employment of these mechanisms may be based on the disparate binding cooperativities of the monomer/activator and dimer/inhibitor forms of its Rep protein, π.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ori, origin of replication; Rep, replication initiator; bp, base pair; wt, wild type; WH, winged-helix; IHF, integration host factor.

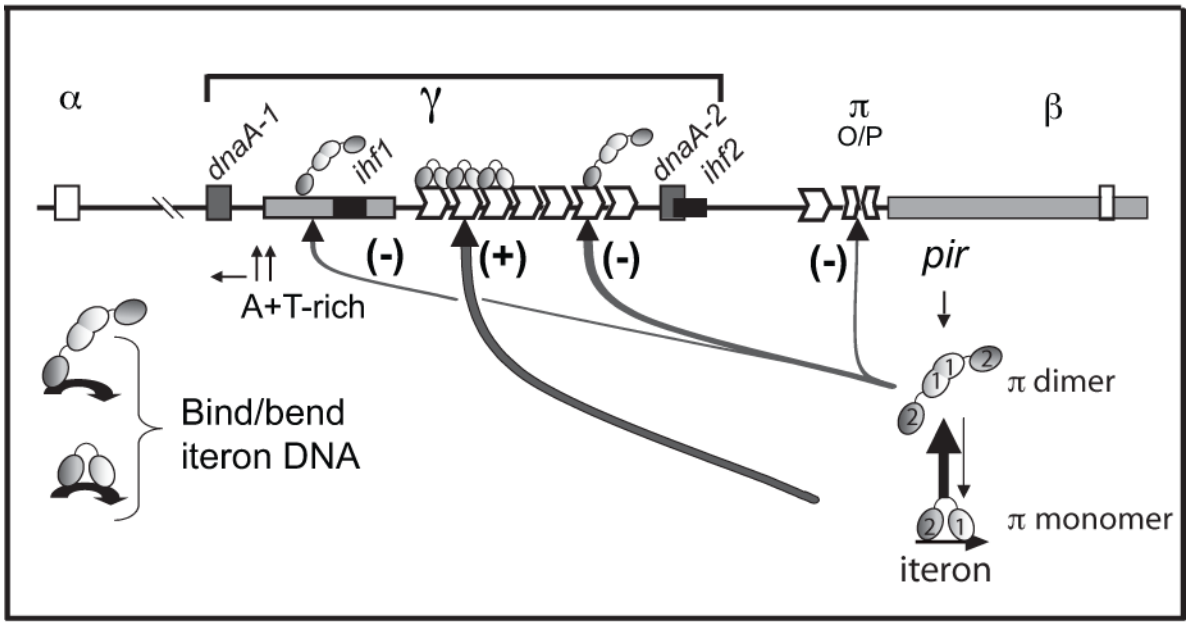

Figure 1.

Plasmid-encoded elements involved in the regulation of replication of γ *ori* plasmids. A physical map of the replicon of R6K showing the relative locations of the seven tandem iterons of γ *ori*, the single iteron and the half-iteron of α *ori* and β *ori*, respectively, the inverted half-repeats in the operator site adjacent to the *pir* gene, two DnaA sites, the integration host factor (IHF) site, the A+T-rich region of γ *ori*, and different paths of *ori* activation and inhibition by π monomers and dimers, respectively. Symbols representing monomers and dimers are labeled and the WH1 and WH2 recognition helices of both monomers and dimers are represented by '1' and '2', respectively. Shading indicates that two monomer subunits of a dimer make head-to-head contacts while two monomers bound to two tandem iterons are proposed to make head-to-tail contacts. A monomer contacts the iteron with both WH1 and WH2[24] while a dimer most likely contacts the iteron only with WH2 of one of the two subunits.

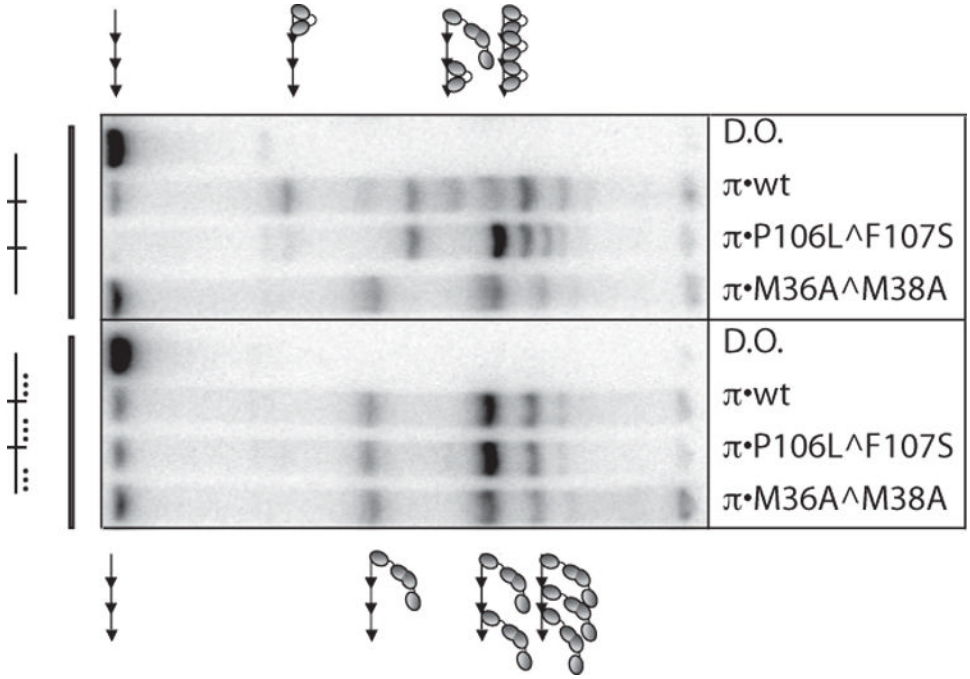

Figure 2.

In vitro binding patterns of π•wt to three tandem iterons with and without mutations in the "monomer-only" side of each iteron. Binding assays were performed with a probe containing three wt iterons in the left four lanes and three monomer-deficient iterons (mutations in the 14th, 17th, and 18th, bp) in the right four lanes. These iteron mutations are depicted by (…). π variants are labeled. DNA probe preparation and gel shift titrations were carried out exactly as previously described[33] except that: 110 pg labeled iteron-containing probe was used in the binding reactions and Promega (Madison, WI) 6X loading dye was added prior to loading the gel. Probe sequences and construction are described in Table 1 of the Supplementary Materials. 200 ng of π was added to each binding reaction. His π•WT, His-π•P106L^F107S, and His-π•M36A^M38A were purified as described.[38,39] D.O. is DNA only. Symbols representing monomers and dimers of π are the same as in Figure 1.

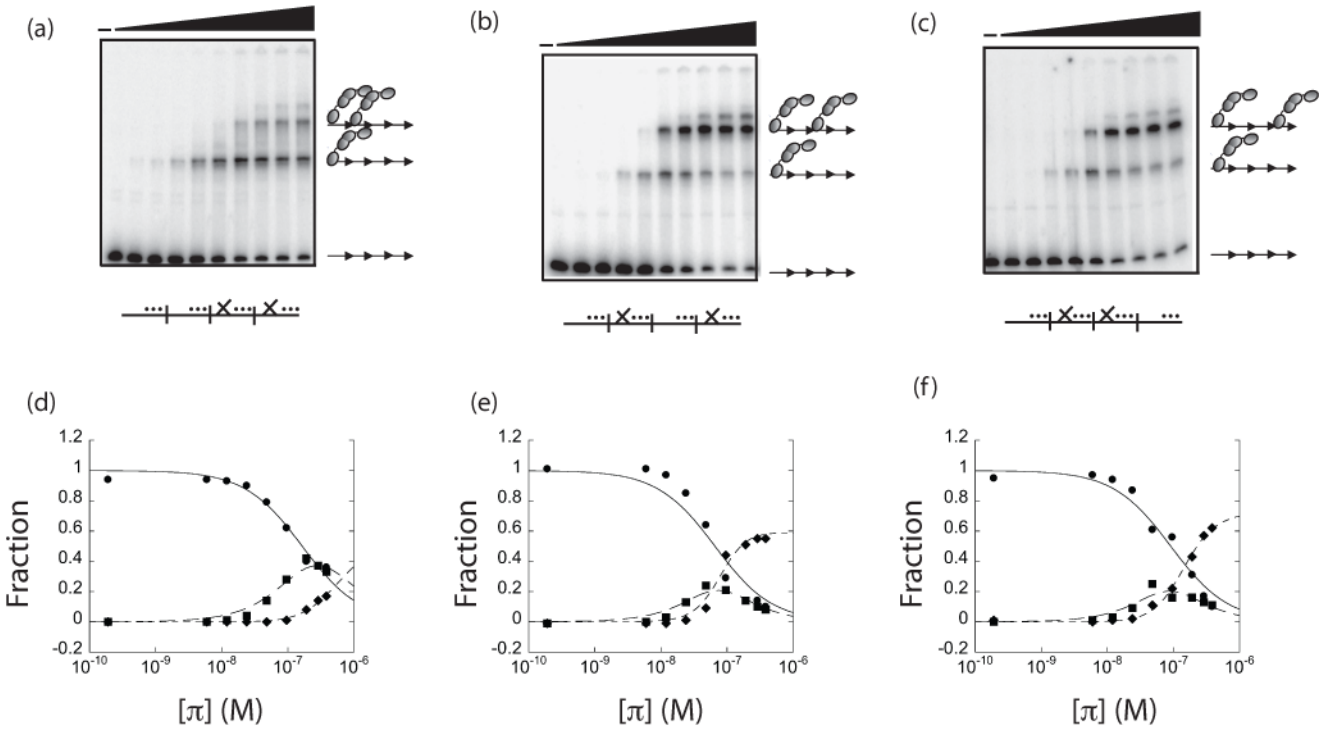

Figure 3.

π binds with greater cooperativity to alternate iterons than adjacent iterons *in vitro*. (a–c) Gel shift titrations of purified π with the depicted probes. (_…) represents an iteron with mutations only in the 14th, 17th, and 18th iteron, which is dimer proficient and monomer deficient. (X…) represents an iteron with mutation in the 7th, and 9th bp as well as the 14th, 17th, and 18th, bp, which cannot bind monomers or dimers. The first lane is DNA only. Black triangles represent increasing levels of π•wt, starting with 3.12 ng (in a 15 □L reaction) and doubling for each lane. DNA probe preparation and gel shift titrations were carried out exactly as previously described[33] except that: 110 pg labeled iteron-containing probe was used in the binding reactions and Promega (Madison, WI) 6X loading dye was added prior to loading the gel. Arrows represent iterons. Gray double-ovals represent π dimers. (d–f) Quantification of gel shift titration data in panels (a–c), respectfully. The fraction of the total radioactivity as free DNA (circles), DNA containing a single π dimer (squares), and DNA containing two π dimers (diamonds) was quantified by fitting data from the gel shift titrations to the following equations using KaleidaGraph software (Reading, PA). The following equations were based on a modified statistical mechanical approach.[33,35,40]

$$\theta_0 = P_0 + (P_{max} - P_0) \cdot 1/Z \quad \text{(Eq. 1a)}$$

$$\theta_1 = P_0 + (P_{max} - P_0) \cdot K_1 L/Z \quad \text{(Eq. 1b)}$$

$$\theta_2 = P_0 + (P_{max} - P_0) \cdot K_2 L^2/Z \quad \text{(Eq. 1c)}$$

θ_o,θ_1,and θ_2 are fractions of free DNA, single dimer complex, and two dimer complexes, respectively. P_0 and P_{max} are the baseline and maximum fraction for a given titration. Z is the binding polynomial and is equal to $1 + K_1L + K_2L^2$. L is protein concentration, $K_1=(k_1 + k_2)$ and $K_2=(k_1k_2k_{12})$. k_1 and k_2 are the binding affinity constants for the first iteron and the second iteron of the 2-iteron complex, and k_{12} is the cooperativity coefficient describing the interaction of protein molecules occupying both sites. Broken, dotted and continuous lines correspond to

the best fit of the data for equations (1a–1c), respectively. Once K_1 and K_2 were obtained from the least squares linear regression analysis, k_{12} was derived by a few simple rearrangements, as described previously.[33]

$$k_{12}=(4K_2)/K_1^2 \quad \text{(Eq. 2)}$$

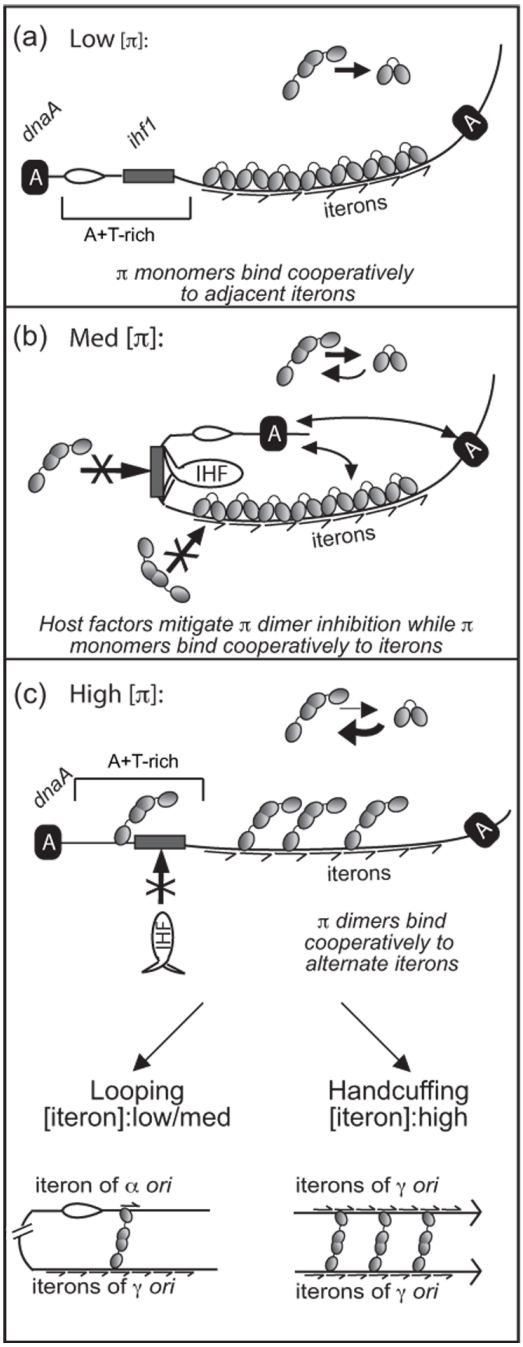

Figure 4.

Proposed role of the disparate cooperative binding properties of π monomers and dimers in the regulation of γ *ori* replication. Symbols representing monomers and dimers of π are the same as in Figure 1. Iterons are depicted by half-arrows. DnaA is labeled as 'A'. Full arrows represent contacts between different molecules of DnaA and between DnaA and π. The open complex is shown as a bubble. (a), (b), and (c) represent a model for the interactions that occur between π, iteron DNA, and host factors at low, medium, and high levels of intracellular π, respectively. In (a), the host factors IHF and DnaA are not needed at low concentrations of π and monomers of π bind cooperatively to all seven iterons, facilitating γ *ori* activation. In (b), as π concentration increases, IHF and DnaA help to mitigate the inhibitory effect of πdimers, allowing monomers

of π to bind cooperatively to all seven iterons and facilitate γ *ori* activation. In (c), at high π concentrations, the dimer out-competes IHF for the *ihf1* site and occupies alternate iterons of γ *ori*. The result may be intramolecular looping or intermolecular handcuffing, depending on the concentration of iteron DNA. This model is explained in more depth in the Discussion section.