Cooperative binding of initiator protein to replication origin conferred by single amino acid substitution

Marcin Filutowicz*, Dona York and Igor Levchenko

Department of Bacteriology, University of Wisconsin, Madison, WI 53706, USA

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ABSTRACT

The replication initiator protein π of plasmid R6K binds seven 22 bp direct repeats (DR) in the γ origin. The π protein also binds to an inverted repeat (IR) in the operator of its own gene, *pir*, which lies outside the γ origin sequences. A genetic system was devised to select for π protein mutants which discriminate between IR and DR (York *et al.*, *Gene (Amst.)* 116, 7–12, 1992; York and Filutowicz, *J. Biol. Chem.* 268, 21854–21861, 1993). From this selection the mutant π S87N protein was isolated which is deficient in repressing the *pir* gene's expression because it cannot bind to IR at the *pir* gene operator. Remarkably, we discovered that π S87N binds to DR cooperatively under conditions where wt π binds independently. Moreover, the π S87N is more active as a replication initiator *in vivo* when supplied at the same level as wt π . Quantitative binding assays showed that both wt π and π S87N bind a DNA fragment containing a single DR unit with a similar affinity ($K_d = 0.3 \times 10^{-12}$ M). Thus, cooperativity of π S87N is most likely achieved through altered interactions between protomers bound at adjacent DR units.

INTRODUCTION

It is remarkable that some replication initiator proteins and prokaryotic transcriptional regulators can bind IR as well as DR sequences (1–9). The molecular mechanism enabling a protein to bind sites of such fundamentally different arrangement is not known. It is also not known in many cases whether the binding of multiple copies of these proteins to repeats is independent, cooperative or antagonistic, and what role the binding mode plays in the control of replication initiation and transcription.

The plasmid R6K provides an opportunity to study these important questions since R6K π protein functions as both regulator of replication and repressor of transcription (4,5,10–14). These two functions can be studied separately because they depend on π binding to the two similar but non-overlapping families of sequence repeats (Fig. 1). Seven 22 bp DR are present in the γ origin of plasmid R6K. An additional copy of the 22 bp DR is present in the regulatory segment of

the *pir* gene that encodes π protein. However, the functional core of the *pir* operator is formed by a 9 bp IR separated by an 11 bp spacer (15). Binding of π protein to the DR is required for localization and activation of the γ origin (3). Binding of π to the IR is required for autoregulation of π synthesis at the transcriptional level (15). In addition, π inhibits DNA replication at elevated levels by an unknown mechanism (13,14).

A genetic system was devised to select for π protein mutants which discriminate between IR and DR (15,16). The properties of a mutant π protein (S87 changed to N87) selected for its inability to repress the *pir* promoter, suggested that different forms of the protein bind these structurally distinct sites. This mutant π S87N protein (formerly designated π^{87} ; 15) has an altered ability to bind the IR but it, like wt π , binds to the additional copy of the 22 bp DR in the operator of the *pir* gene. Since dimerization properties of π S87N in solution are indistinguishable from that of wt π (15), its altered discrimination reflects a different type of change. Moreover, π S87N can still activate the γ origin and inhibits replication *in vivo* less severely than wt π (15). In this study, we asked whether wt π and π S87N proteins differ in any aspect of binding to γ origin DNA and replication initiator activities.

MATERIALS AND METHODS

Plasmid constructs

Plasmid pMF34 contains the entire γ origin with the seven 22 bp DR (17). Six of seven DR have been deleted in construct pMF239, leaving a single wt 22 bp DR in the context of the rest of the γ origin (18).

Plasmids pMM17 Δ 17wt *pir* and pMM17 Δ 17*pir*87 used in the purification of π protein have been described (19). In these plasmids the expression of π protein is under the control of the IPTG-inducible *P_{tac}* promoter (19). Plasmid RK526 is a kanamycin-resistant R6K derivative carrying the γ origin (10).

Protein purification

The wt π and π S87N proteins were purified from IPTG-induced cultures of JM105 containing pMM17 Δ 17wt *pir* and pMM17 Δ 17*pir*87. Proteins were purified using a previously published procedure (17) with modifications (15).

*To whom correspondence should be addressed

Figure 1. DR and IR repeats the replication region of plasmid R6K (15,23). The cluster of seven tandem 22 bp direct repeats in the γ origin is shown. The eighth 22 bp DR and the two 9 bp IR in the *pir* operator are indicated by arrows. Area of RNA polymerase interaction is bracketed, with -35 and -10 hexamers of the *pir* promoter (P3) overlined (4,5). 115 bp lie between the seven repeats at the γ origin and the eighth DR in the operator. Each repeat contains the TGAG motif in which G's at the second and fourth position have been shown to be important for π binding (3).

Gel retardation assays

CsCl-purified plasmid DNA (pMF34 or pMF239) was digested with *Hind*III, phenol-treated, precipitated with isopropyl alcohol, washed with ethanol and dried. π -DNA complexes were assembled using π -dilutions in TBE buffer (50 mM Tris–HCl, 45 mM boric acid, 1.4 mM EDTA–Na₄; pH 8.3) from protein stocks. Conditions of electrophoresis are described in the figure legends.

DNase I protection assays

DNase I protection was performed with a fragment obtained from plasmid pMF239 5'-end labeled at the *EcoRI* site and recut with *SaII*. Ninety nanograms of the labeled fragment were combined with either wt π and π S87N proteins. Binding reaction was carried out at 25°C in buffer containing the following components: 25 mM Tris pH 7.5; 5 mM Mg-acetate and 100 mM K-glutamate. The DNase I cleavage reaction and gel processing were as described previously (15).

RESULTS AND DISCUSSION

An enhanced replication initiator activity of π S87N *in vivo*

Although the transcriptional repressor function of π S87N is impaired, its initiator function is not (15). However, some aspect of the initiation dependent on π S87N must be different from wt π , since the γ origin can be activated even at elevated levels of π S87N; the equivalent level of wt π completely inhibits replication (15).

The reduced ability to inhibit replication is the characteristic feature of several previously characterized ‘copy up’ mutations in the *pir* gene (13). Since the *pir87* mutation had occurred in the region in which a majority of copy up mutations map, we asked whether the *pir87* mutation increases the plasmid copy number as well. This was done with a kanamycin resistant derivative of plasmid R6K containing the γ origin (pRK526); its replication depends on π protein provided in *trans* (10). As shown in Fig. 2 with π S87N the copy number of the pRK526 plasmid increased approximately three-fold above that observed in cells producing the equivalent level wt π protein (15). It should be emphasized that although π S87N is altered in autoregulation

Figure 2. Levels of plasmids pRK526 (γ origin) and pMM17 Δ 17 (*pir*-containing helper) in JM105 host grown in the absence of IPTG. (In the presence of IPTG pRK526 cannot replicate with the ω pt but it can with π 587N; 15). Five milliliters of overnight cultures of each strain were harvested and DNA was isolated according to Birnboim and Doly (25). One-third of the isolated plasmid DNA was digested with *Bgl*II as well as 3 μ g of purified pRK526 DNA as a control. Three and one half (3.5 $\times$) and 1 μ l (1 $\times$) of each 20 μ l digest were loaded and 5 μ l of *Bgl*II digested pRK526 control. The location of plasmids pRK526 and pMM17 Δ 17 are indicated by arrows. The photograph is a reverse image of ethidium bromide stained gel.

its intracellular level is experimentally adjusted via the artificial IPTG-inducible *P_{tac}* promoter (15). In the absence of IPTG, pMM17Δ17wt*pir* and pMM17Δ17*pir*87 produce similar levels of π (15). Therefore, we conclude that the *pir*87 mutation has copy up phenotype and that this phenotype is not caused by an altered level of π protein.

π S87N mutant protein binds seven DR in a single-step-reaction while wt π binds in multiple steps

A similar binding of π S87N and wt π to the 22 bp DR in the operator of P3 promoter suggested that their binding to seven DR in γ origin might also be similar (15). We addressed this question directly by examining the binding of wt π and π S87N

Figure 3. Binding of wt and π S87N proteins to a DNA fragment containing DR as determined by mobility shift assay. Both proteins were overproduced from constructs containing *Ptac* promoter and purified as described in Materials and Methods. Binding reactions contained 0.4 μ g of a *Eco*RI–*Pst*I (*RI*, *Pst*) digest of plasmid pMF34 (including vector and origin fragments) as shown in (A), and the indicated amounts of wt π or π S87N proteins (B) were assembled in 2.5 μ l of TBE buffer. Each sample, shown in (B), was equilibrated for 10 min at 25°C, and after addition of 2.5 μ l of native tracking dye [20% sucrose (w/v), 1 mM EDTA- Na_2 , 20 mM Tris–HCl (pH 7.5), 0.01% (w/v) xylene cyanol and 0.01% (w/v) bromophenol blue] electrophoresed at 13 V/cm for 1 h in a 6% polyacrylamide.

Figure 4. Interaction of wt π and π S87N with a single DR as determined by DNase I footprinting. DNase I footprinting was performed with 90 ng of ^{32}P -labelled *Eco*RI/*Sa*I fragment isolated from plasmid pMF239. Plasmid pMF239 contains a precise deletion of six DR (18). The amounts of each protein are indicated above the photograph. The nucleotide positions (23,24) within the DR are indicated by horizontal arrows with the location of the deletion designated by Δ . The vertical line designates the position of the 22 bp DR.

protein to a γ origin fragment using a gel mobility shift assay. As shown in Fig. 3, and as previously reported (17), binding to a γ origin fragment containing the seven 22 bp DR occurs in multiple discrete steps as increasing amounts of wt π protein are added. However, a remarkable difference is seen with the mutant π S87N protein: it produces only the fully bound DNA species without any intermediate-bound DNA species detected. Thus, these two proteins differ in binding mode to the DR in the γ origin; wt π binds in multiple steps while π S87N binds in a single step. These data suggest that π S87N binds the seven

Figure 5. Quantitative assay of binding of wt π and mutant π S87N to a DNA fragment containing a single 22 bp repeat as determined by a mobility shift assay in a polyacrylamide gel. In the mobility shift assay each binding reaction was assembled with 6 fM of ^{32}P labelled DNA of a 161 bp *Hind*III fragment obtained from plasmid pMF239 and 0.1 μ g of poly(dI-dC) from Pharmacia. The binding reactions were assembled in 4 μ l of Tris–Borate–EDTA buffer (see Fig. 3 legend) with the indicated amounts of wt π and mutant π S87N. Reactions were equilibrated for 20 min at 25°C and 0.5 μ l of native tracking dye was added. The 6% acrylamide gels (acrylamide to bis-acrylamide weight ratio 30:1) with TBE buffer were pre-electrophoresed to constant current at 6 V/cm with buffer recirculation. Samples were loaded on to gel running at 15 V/cm without interrupting the current. After marker dyes entered the gel, the current was reduced to 6 V/cm and gel was run at 4°C for 2 h. Gels were fixed with 10% acetic acid and dried. Autoradiogram was obtained by using OG Kodak film for 24 h at –70°C. 50% of unbound DNA fragment were calculated from the autoradiograms. Autoradiograms were scanned with Hoeffer Scientific Instruments scanning densitometer GS300 using program GS370 Data system (Apple Macintosh version).

DR in a highly cooperative manner. In contrast, wt π binds independently. The cooperative binding appears to depend on the presence of multiple DR sequences because oligomerization of π S87N protein does not occur in the absence of DNA (15) or in the presence of a single 22 bp DR unit (see section below).

Wt π and π S87N have similar affinity for a single DR unit

Are there differences in the mechanism of DR sequence recognition by wt π and π S87N? To answer this question a DNase I protection assay was performed with a DNA fragment isolated from plasmid pMF239 that contains a single 22 bp DR unit (see Materials and Methods). Figure 4 shows that both proteins produce previously characterized patterns of protection and enhancements (17) when they are provided at the similar levels. Therefore, the fundamental change in the binding mode of π S87N is not caused by altered recognition of the 22 bp DR unit.

To determine if there were differences in the affinity and in the oligomerization state of wt π and π S87N bound to DNA, we performed a quantitative gel retardation assay using a DNA fragment containing only one 22 bp repeat unit. As shown in Fig. 5, wt π and π S87N caused a similar retardation pattern when a fragment containing a single 22 bp repeat unit was used. The reason for the formation of two complexes (C1 and C2, Fig. 5) with the fragment containing a single repeat unit is unknown. Regardless of this uncertainty, it is clear that, under the experimental conditions employed, the extent of oligomerization of wt π and π S87N proteins bound to a 22 bp repeat is the same. These results are also consistent with the identical state of oligomerization of the wt π and π S87N in solution (15).

From the range of protein concentrations required for the titration of a repeat-containing DNA fragment, it is also evident that the apparent binding constant for a single 22 bp repeat unit is virtually identical for wt π and π S87N proteins ($K_d = 0.3 \times 10^{-12}$ M; Fig. 5 bottom). These data are consistent with DNase I footprinting experiments which showed that the amounts of wt π and π S87N required for binding the 22 bp repeat in the *pir* operator are similar as well, even though footprints were performed in the absence of a nonspecific competitor DNA (Fig. 4). Therefore, the fundamental change in the binding mode of π S87N does not seem to be caused by its altered affinity for the 22 bp repeat unit.

Two points require additional comments. First, considering that the mutant binds cooperatively, it is unusual that the wt π and π S87N show similar half-maximal binding to the seven DR. In a case like this, the intrinsic binding affinity for a single DR would be expected to be lower (by the cooperativity factor) for π S87N. We cannot explain this observation at the present time. Second, it remains to be determined if the *pir*87 mutation confers cooperativity on π protein or greatly enhances so far undetected cooperative interactions between wt π protomers. Addressing this question requires an investigation of the effect of various electrolytes on binding mode, because the difference described here between wt π and π S87N was seen in only one buffer (TBE).

As we already pointed out the molecular mechanism enabling a protein to recognize related DNA sequences arranged as DR or IR is not known. However, to explain such an ability of AraC of *Escherichia coli*, it was proposed that this protein might assume either head-to-tail or head-to-head conformations by using a flexible protein spacer (20). The nature of conformational change induced by the *pir*87 mutation is not known. However, taking into consideration the structural requirements permitting binding of a protein to IR and either cooperative or independent binding

to DR it seems plausible that π may also exist in either head-to-tail or head-to-head dimeric conformations. Transition of one form into another form can, perhaps, be regulated depending on intracellular conditions. Interestingly, we have recently shown that both dimerization and dimer stability are determined by the N-terminal domain of π (21) and we have speculated that π dimerization could be mediated by two putative interfaces: the leucine zipper and RGD motifs (15,21). In this scenario, the π S87N might be altered in one of these interfaces since the S87 is separated by four amino acids from the RGD motif.

We have recently discovered another IR in the γ origin (26); TGAGAG-11nt-TTCTCA at the R6K coordinates -71 to -49 (23,24). DNase I footprinting experiments revealed that the wt π can bind this site, but π S87N cannot, even though both proteins can bind to the seven DR present on the same DNA fragment (I.Levchenko, F.Wu and M.Filutowicz, manuscript in preparation). Thus, π S87N cannot recognize IR in the two distinct sequence contexts.

What could be the biological significance of our finding? As mentioned, π protein is multifunctional: it activates γ origin as well as inhibits replication and autoregulates its own synthesis. An attempt to explain these functions by biochemically distinct forms of π protein has been made (22). Our findings suggest that π molecules responsible for origin activation may differ structurally from those responsible for inhibition of replication and repression of the *pir* gene expression. The fact that π S87N is more active than wt π as replication initiator and is deficient in autoregulating its own expression is consistent with the two forms of π protein model.

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