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Dual nucleotide specificity determinants of an infection aborting anticodon nuclease

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ABSTRACT

The anticodon nuclease (ACNase) PrrC is silenced by a DNA restriction-modification (RM) protein and activated by a phage T4-encoded restriction inhibitor. The activation is driven by GTP hydrolysis while dTTP, which accumulates during the infection, stabilizes the active form. We show here, first, that the ABC-ATPase N-domains of PrrC can accommodate the two nucleotides simultaneously. Second, mutating a sequence motif that distinguishes the N-domain of PrrC from typical ABC-ATPases implicates three residues in the specificity for dTTP. Third, failure to bind dTTP or its deprivation hastened the centrifugal sedimentation of PrrC, possibly due to exposed sticky PrrC surfaces. Fourth, dTTP inhibited the GTPase activity of PrrC, probably by preventing GDP from leaving. These observations, correlated with relevant traits of a related ACNase, further suggest that PrrC utilizes GTP at canonical ABC-ATPase sites and binds dTTP to distinct sites exposed upon disruption of the ACNase-silencing interaction with the RM partner.

Introduction

The phage T4-excluding anticodon nuclease (ACNase) PrrC is silenced in its uninfected *Escherichia coli* host by a type I DNA restriction-modification (RM) protein and turned on by the T4 DNA restriction inhibitor Stp. The consequent cleavage of $tRNA^{Lys}$ 5′ to the anticodon could block the synthesis of the late T4 proteins but the damage is reversed and the infection rescued by T4-encoded tRNA repair enzymes (Kaufmann, 2000). *In vitro* activation of the latent ACNase (PrrC/RM complex) requires, apart from the DNA restriction inhibitor Stp, the hydrolysis of GTP and the presence of dTTP or its non-hydrolysable analog dTMPPCP. In contrast, free PrrC exhibits overt ACNase activity that is indifferent to GTP but highly unstable without dTTP or TMPPCP (Amitsur et al., 2003). The biological significance of the protection that dTTP imparts is hinted at by the accumulation of dTTP in the T4 infected cell. Namely, the increased dTTP level, which is needed for faithful replication of the AT-rich T4 DNA (Sargent and Mathews, 1987) is also coopted by the infected host for stabilizing its activated ACNase (Klaiman and Kaufmann, 2011).

The N-proximal 284 residues of the 396-residue long *E. coli* PrrC constitute an ATP Binding Cassette (ABC)-ATPase domain (George and Jones, 2013) (Fig. 1). This domain has been implicated in the respective ACNase-activating and ACNase-stabilizing effects exerted by GTP and dTTP (Blanga-Kanfi et al., 2006). The remaining part, considered the ACNase domain (Meidler et al., 1999; Jiang et al., 2001, 2002) has been assigned to the HEPN superfamily, along with other infection-aborting ribotoxins (Anantharaman et al., 2013). According to one view, PrrC is a dimer of dimers whose N-domains dimerize head-to-tail like typical ABC-ATPases while the C-domains interface in parallel (Blanga-Kanfi et al., 2006; Klaiman et al., 2007). According to another view, PrrC acts as a dimer whose C-domains do not interface (Meineke et al., 2011).

The ABC-ATPase superfamily comprises motor components of membrane spanning transporters and soluble proteins engaged in DNA repair, translation and other activities. ABC-ATPases bind two ATP or GTP molecules to cognate sites formed between anti-parallel dimerization interfaces and exert their function by power strokes of nucleotide hydrolysis (Hopfner et al., 2000; Lammens et al., 2004; Smith et al., 2002; Chen et al., 2003). The nucleotide binding sites (NBS) arise between a sub-domain containing the Walker A and B motifs of one subunit and the Signature sub-domain of the other (Procko et al., 2009). In one current model the two nucleotide molecules are bound simultaneously but are hydrolyzed sequentially. In another model the binding and hydrolysis occur first at one NBS and then at the second (George and Jones, 2013). The nucleobase specificity of certain ABC-ATPases is ascribed to vicinal aromatic and

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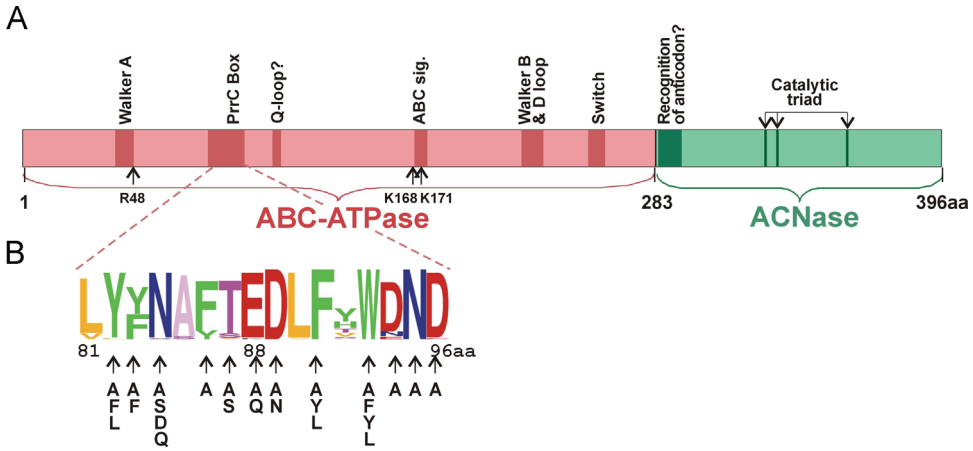

Fig. 1. Functional organization of PrrC. (A) PrrC is divided into ABC-ATPase and ACNase domains (pink and green rectangles, respectively). Canonical ABC-ATPase motifs including a putative Q-loop, PrrC Box, putative anticodon recognition region and catalytic ACNase triad (residues 320, 324 and 356) are highlighted in darker colors. The estimated end of the N-domain is at residue 283 instead of the previously considered residue 265 (Blanga-Kanfi et al., 2006). Three residues within or near ABC-ATPase motifs distinguish the N-domain of PrrC from typical ABC-ATPase, R48, K168 and K171. These residues, indicated by upward pointing arrows, were replaced in this work by T, N and N, respectively. (B) WebLogo presentation (Crooks et al., 2004) of the PrrC Box motif (PrrC residues 81–96). PrrC Box residues mutated in this work are indicated by upward pointing arrows. The replacements introduced are indicated below these arrows.

polar residues located upstream of the Walker A motif (Guo et al., 2006; Ambudkar et al., 2006).

To reconcile the presumed GTP/dTTP specificity of the N-domains of PrrC with the ABC-ATPase canon we considered the possibilities that the ABC-ATPase sites of PrrC (i) uniformly switch between GTP and dTTP binding modes, (ii) stably segregate into GTP-utilizing and dTTP-binding types or (iii) utilize only GTP while the binding of dTTP is delegated to sites formed by different sequence motifs that set the N-domain of PrrC apart from typical ABC-ATPases. We suspected that such a motif could be the aromatic/polar stretch of 16 residues located between the Walker A and Q-loop motifs (PrrC Box, Fig. 1). Its composition, conservation and unique presence in PrrC suggested involvement in the specificity for dTTP, although added or alternative tasks such as interfacing the RM protein or dimerizing the dimers were not excluded.

The homologous ACNase RloC (Davidov et al., 2008; Klaiman et al., 2012', 2014) was also expected to provide clues to how PrrC interacts with its nucleotide ligands. Like PrrC, RloC comprises ABC-ATPase and ACNase N- and C-domains. However, unlike PrrC, RloC has an internal ACNase switch that comprises a dsDNA break (DSB) sensor coupled to the intrinsic ATPase. Importantly, several lines of evidence have suggested that sustained activity of the RloC ACNase requires that the protein retain the nucleotide it hydrolyzed and remain tethered to the activating DNA termini. Since the overt ACNase activity of PrrC is stabilized not only by dTTP but also by GDP (Amitsur et al., 2003), we assumed that the two nucleotides cooperate in this function similar to the synergizing RloC ligands.

The above assumptions about the interaction of PrrC with its disparate nucleotide ligands were addressed by nucleotide–protein UV-crosslinking, mutagenesis, ACNase and GTPase assays and centrifugal sedimentation. The data obtained, correlated with relevant RloC traits, underlie a model where PrrC utilizes GTP at its canonical ABC-ATPase sites and binds dTTP to other sites that are exposed upon disruption of the ACNase-silencing interaction with the RM protein.

Results

GTP and dTTP may occupy the N-domains of PrrC simultaneously

PrrC is inherently unstable in the absence of the RM protein. This has been indicated by the failure to detect the ACNase activity of PrrC when expressed in *E. coli* at a low, physiological-like level without the RM partner. In contrast, when PrrC is similarly expressed in the presence of the RM partner it is captured in the latent, activatable ACNase complex (Amitsur et al., 2003). When PrrC is overexpressed it elicits strong ACNase activity that limits its expression. This limitation and the inherent instability of PrrC impeded the isolation of the *wt* protein or highly active mutants of it.

Therefore, *in vitro* studies on PrrC depended on the partially active mutant PrrC^{D222E} that can be expressed to a higher level and purified in active form (Blanga-Kanfi et al., 2006). UV-crosslinking and ACNase assays revealed that PrrC^{D222E} bind GTP and dTTP with respective sub-mM- and μM-range affinities (Amitsur et al., 2003; Blanga-Kanfi et al., 2006). However, since Asp^{222} equivalents of other ABC-ATPases are implicated in catalytic and structural roles (Smith et al., 2002; Zaitseva et al., 2005; Oldham and Chen, 2011) it was not clear if the nucleotide binding attributes of PrrC^{D222E} faithfully represent those of the *wt* protein. Due to this reason and to test the premise that the N-domain of PrrC encodes the dual GTP/dTTP specificity, the nucleotide binding attributes of PrrC^{D222E} were compared to those of *wt* and D222E alleles of an N-domain construct.

The N-domain of PrrC was previously assumed to span residues 1–265 (Blanga-Kanfi et al., 2006). However, comparing the solubility and protein yields of constructs containing from 265 to 284 N-proximal residues suggested that only the largest represents the entire N-domain (Fig. S1A). Moreover, inspection of the crystal structures of the ABC-ATPases Rad50 and RecF (Hopfner et al., 2000; Koroleva et al., 2007) suggested that their C-terminal β-hairpin (Fig. S1B and C) matches PrrC residues 269–284 (Fig. S1D). PrrC$_{1-284}$ was employed therefore as the standard N-domain construct (henceforth PrrC-ND). The sedimentation velocity and glutaraldehyde (GA)-mediated crosslinking pattern of PrrC-ND suggested an oligomeric, dimer of dimers structure (Fig. S2), as proposed for the full-sized PrrC^{D222E} (Blanga-Kanfi et al., 2006).

Irradiating PrrC^{D222E}, PrrC-ND or PrrC^{D222E}-ND at 254 nm in the presence of increasing levels of [α-^{32}P]GTP yielded in each case a dose–response commensurate with EC$_{50}$ of ~0.3 mM (shown for PrrC^{D222E} in Fig. 2A lanes 1–5, for PrrC-ND in lanes 6–10). The decline at the higher GTP levels was attributed to UV-light quenching by GTP rather than decreased GTP binding. Namely, a sigmoidal dose–response characterizes the activation of the latent PrrC ACNase by GTP (Amitsur et al., 2003) and the binding of GTP by different PrrC alleles that was determined by label-free microscale thermophoresis (MST) (Fig. 2B). MST exploits changes