Doc of Prophage P1 Is Inhibited by Its Antitoxin Partner Phd through Fold Complementation*‡§

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Prokaryotic toxin-antitoxin modules are involved in major physiological events set in motion under stress conditions. The toxin Doc (death on curing) from the phd/doc module on phage P1 hosts the C-terminal domain of its antitoxin partner Phd (prevents host death) through fold complementation. This Phd domain is intrinsically disordered in solution and folds into an α-helix upon binding to Doc. The details of the interactions reveal the molecular basis for the inhibitory action of the antitoxin. The complex resembles the Fic (filamentation induced by cAMP) proteins and suggests a possible evolutionary origin for the phd/doc operon. Doc induces growth arrest of *Escherichia coli* cells in a reversible manner, by targeting the protein synthesis machinery. Moreover, Doc activates the endogenous *E. coli* RelE mRNA interferase but does not require this or any other known chromosomal toxin-antitoxin locus for its action in vivo.

Small operons encoding a toxin and its antitoxin are common in the genomes of bacteria and archaea and are also found on certain plasmids and bacteriophages. These so-called toxin-antitoxin (TA) modules have been proposed to regulate the pace of metabolism and may induce a state of dormancy in case of nutritional stress (1–3). TA modules are highly abundant in opportunistic pathogens such as *Mycobacterium tuberculosis* (4), and their presence has been linked to persistence (5).

On plasmids, TA modules act as addiction systems, aiding plasmid maintenance in the bacterial population by post-segregational killing (6), filling in a function related to apoptosis and programmed cell death in eukaryotes (7). Related effects have been observed for chromosome-located TA systems as some of them have been shown to diminish large scale genome reductions in the absence of selection (8). In the presence of the plasmid, both toxin and antitoxin are expressed, leading to a steady state equilibrium where the antitoxin counteracts the effect of the toxin. In its free state, the antitoxin, usually a modular protein that contains a functional intrinsically disordered region (9–11), is under constant proteolytic attack. The toxin-antitoxin complex acts as an autorepressor for the TA operon, ensuring that only small amounts of the proteins are present in the cell. Upon plasmid loss, the antitoxin is degraded by a specific intracellular protease, releasing the toxin. Without the possibility of replenishing the antitoxin population, the toxin action becomes irreversible, resulting in cell death.

The phd/doc operon encodes a TA module aiding the maintenance of the plasmid-prophage P1 in *Escherichia coli* (12). Doc is an inhibitor of translation elongation through its association with the 30 S ribosomal subunit in a way similar to the antibiotic hygromycin (13). The action of Doc is suppressed by the antitoxin Phd, which consists of two domains. Its C-terminal domain (residues 52–73) harbors the interaction site with Doc and on its own prevents Doc-mediated growth arrest (14, 15). The N-terminal region (residues 1–51) of Phd is a dimerization-DNA-binding domain that binds to the operator site of the phd/doc operon. Phd forms a heterotrimeric complex with Doc (16). Operator binding and repression of the phd/doc operon by Phd are enhanced by the presence of Doc in a cooperative manner (17–19).

Here we present the crystal structure of a non-toxic version of Doc (DocH66Y, an H66Y mutant of Doc) in complex with the C-terminal domain of Phd (Phd52–73Se, a peptide corresponding to residues 52–73 of Phd with Se-Met substituted for Leu-52 and Leu-70) and provide further information on the interplay between Doc and endogenous chromosomal TA modules. The structure reveals a new all α-helical fold, leads to new insights into the

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‡ The atomic coordinates and structure factors (code 3dd7) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Materials and Methods, four supplemental figures, two supplemental tables, and supplemental references.

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2. The abbreviations used are: TA, toxin-antitoxin; PDB, Protein Data Bank; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

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molecular action of Doc and its interaction with Phd, and suggests a possible origin for the phd/doc module.

EXPERIMENTAL PROCEDURES

Crystal Structure Determination—Purification of DocH66Y and crystallization of DocH66Y and of its complex with Phd52–73Se (C-terminal 22 residues of Phd with Leu-52 and Leu-70 substituted to Se-Met) are reported elsewhere. After a failed attempt to phase the structure of DocH66Y-Phd52–73Se using the two Se-methionines present in Phd52–73Se, a crystal was soaked for about 3 min in a cryo-protecting solution (0.2 m NaCl, 0.1 m sodium acetate, pH 4.6, and 35% 2-methyl-2,4-pentanediol) enriched with 1.5 m of NaBr. Data were collected at the K-edge of bromine and at a high energy remote wavelength (Table 1). The data sets were indexed, integrated, and merged using the HKL suite of programs (20). Substructure calculation with ShelxL (21) detected 17 potential bromine sites. Phases were calculated by two-wavelength multiwavelength anomalous dispersion using the software pipeline AutoRickshaw (22). The experimentally phased electron density map allowed automated building of the model with ARP/wARP (23) after 2-fold symmetry averaging of the electron density based upon non-crystallographic symmetry parameters derived from the known heavy atom positions. The resulting model was highly complete and refined using REFMAC5 (24) to an Rfree factor of 19.8% and a conventional R-factor of 18.3%. Details of data collection and refinement statistics are given in Table 1.

CD Spectropolarimetry—Far UV-CD spectra were recorded on a J-715 spectropolarimeter (Jasco). Scans were taken using a 0.1-cm cuvette. The temperature of the cuvette was monitored using a probe, and a water bath was used for maintaining the temperature of the sample constant. The measurements were performed at 298 K, in 50 mM Tris (pH 7.5), 150 mM sodium chloride. Spectra of DocH66Y and Phd52–73Se were taken using a protein concentration of 60 μM. For the spectrum of the complex, DocH66Y and Phd52–73Se were mixed in equimolar ratio to a final concentration of 60 μM of the complex and preincubated for 5 min before taking the spectrum. The mean residue ellipticities ([θ], degrees cm² mol⁻¹) were obtained from the raw data (θ, ellipticity) after correcting for the buffer solution, according to [θ] = θMw/(lnc), where Mw is the molecular weight, c is the mass concentration, l is the optical path length, and n is the number of amino acid residues.

Northern Blotting and Primer Extension Analyses—Strains and plasmids used in this work are described in the supplemental Materials and Methods and are summarized in supplemental Table S1. Cells were grown in LB at 37 °C. At an A450 of 0.5, the cultures were diluted 10 times and grown to an OD of 0.5. Transcription of the toxins was induced by the addition of arabinose to 0.2%. To inhibit translation, chloramphenicol (50 μg/ml) was added. For Northern analysis, total RNA was fractionated by PAGE (6% bis-acrylamide), blotted to a Zeta-Probe nylon membrane, and hybridized with a single-stranded 32P-labeled riboprobe, complementary to the RNA. The radioactive probe was generated using linearized plasmid DNA of pSC333 constructing probes for lpp mRNA. Semiquantitative primer extension analysis was performed essentially according to the method previously described (25). The stop codons of mRNAs originating from pKW254T derivatives were mapped with the primer pKW71D-3#PE, which is complementary to the RNA. The radioactive probe was generated using linearized plasmid DNA of pSC333 constructing probes for lpp mRNA. The complete list of oligonucleotides used is given in supplemental Table S2.

RESULTS

Structure of DocH66Y—Because of difficulties in producing sufficient amounts of wild-type Doc, we used the less toxic mutant H66Y (19) for structure determination. DocH66Y was crystallized in complex with a peptide encompassing the C-terminal 22 amino acids of Phd with Se-Met substituted for Leu-52 and Leu-70 (Phd52–73Se). This fragment was chosen based upon previous work that delineates the toxin-binding domain of Phd (14, 15). The Doc protein shows an all-α-helical fold consisting of six α-helices (Fig. 1). It can be described as the stacking of three consecutive helix-loop-helix elements. Helices α3 and α4 are central in the structure and carry a high proportion of aliphatic side chains to pack α1 and α2 on one side and α5 and α6 on the other.

Multiple sequence alignment of Doc family members reveals a single highly conserved motif, HXFX(D/E)(A/G)K(N/K)G/R. It

| TABLE 1 |
| Data collection and refinement statistics |
| r.m.s., root mean square. |
| | DocH66Y-Phd52–73Se | DocH66Y-Phd52–73Se, Remote |
| Beamline | X12 (EMBL Hamburg) | X12 (EMBL Hamburg) |
| Wavelength (Å) | 0.9189 | 0.9197 |
| Space group | C2 | C2 |
| Unit cell | | |
| a (Å) | 110.9 | 110.9 |
| b (Å) | 38.2 | 38.2 |
| c (Å) | 62.7 | 62.7 |
| β (°) | 99.3 | 99.3 |
| Resolution limits (Å) | 15.0–1.7 | 15.0–1.7 |
| Number of measured reflections | 642571 | 649052 |
| Number of unique reflections | 29371 | 29397 |
| Completeness | 96.7 | 95.7 |
| Rmerge | 0.09 | 0.09 |
| Rmerge | 9.0 | 8.3 |
| Rmerge | 18.2 | 18.2 |
| Ramachandran profile | | |
| Core | 100% |
| Other allowed | 0.0% |
| Outliers | 0.0% |
| r.m.s. deviations | | |
| Bond lengths (Å) | 0.01 |
| Bond angles (°) | 1.24 |
| Number of atoms | | |
| Protein | 2307 |
| Water | 228 |
| Other | 16 |
| B-factors (Å²) | | |
| From Wilson plot | 19.4 |
| All atoms | 22.3 |
| Protein atoms | 20.7 |
| Water atoms | 30.5 |
| Other atoms | 24.5 |
| PDB entry | 3dd7 |
| a Rmerge = Σhkl[Sobs(hkl)−(Σi|Fobs(hkl)|2)1/2]/Σhkl|Fobs(hkl)| |
| b Rmerge = Σhkl|Fobs(hkl)| − k|Fcalc(hkl)|²]1/2/Σhkl|Fobs(hkl)| |

3 A. Garcia-Pino and R. Loris, manuscript in preparation.
FIGURE 1. Structure of Doc. A, a stereo view of the Doc$^{H66Y}$-Phd$^{52–73Se}$ complex. Helices of Doc$^{H66Y}$ are shown in cyan, and loop structures are shown in gray. The α-helices are labeled. The loop α3-α4 containing the conserved sequence motif HXFXID/E(A/G)N(K/G)R is highlighted in red, and its side chains are shown as sticks. Loop α1-α2 is highlighted in blue. The bound Phd$^{52–73Se}$ fragment is shown in yellow.

is located in the loop α3-α4 (residues 66–74) (Fig. 1), and its conformation can be described as two consecutive NEST motifs. These are short structural elements that are often used as anion-binding sites (26) (supplemental Fig. S2a). This sequence motif forms the core of a single patch of conserved surface residues (Fig. 2A) that further includes residues from the loop α1-α2 as well as His-13. The latter plays a structural role for establishing the correct conformation of the conserved sequence motif. In addition, a number of mutations known to eliminate Doc toxicity but that retain co-repression activity (H66Y, H66R, and D70N) map within this motif (Fig. 2B) (19). This is a clear indication for a functional role of this region, likely an interaction site.

Interactions with Phd—The C-terminal domain of Phd on its own is sufficient to protect against Doc (14, 15). Phd$^{52–73Se}$ binds into a groove of Doc$^{H66Y}$ of which helix α4 forms the base and that is flanked by helix α1 on one side and the loop α4-α5 on the other side (Fig. 1A). This binding site is adjacent to the hotspot of conserved residues on the surface of Doc but by itself is not highly conserved (Fig. 2A). The binding groove has an approximate volume of 2050 Å$^3$ and contains a large number of positively charged side chains (Arg-2, Arg-19, Lys-73, Arg-74, Arg-85, and Arg-86) around a hydrophobic center. As such, it forms a positively charged patch on the otherwise negatively charged surface of Doc (Fig. 2C).

Far UV CD experiments show that Phd$^{52–73Se}$ is intrinsically unstructured in its isolated state but gains an appreciable amount of α-helix upon binding to Doc (Fig. 3). In the crystal structure, Phd$^{52–73Se}$ adopts an α-helical conformation when bound to Doc and shields about 820 Å$^2$ of the Doc surface from the solvent. The interactions between Phd$^{52–73Se}$ and Doc$^{H66Y}$ are mediated entirely by side chain atoms (Fig. 4A). A kink in the α-helix divides Phd$^{52–73Se}$ into a hydrophobic N-terminal segment (residues 54–62) and a predominantly negatively charged C-terminal segment (residues 64–73) (Fig. 4B). In its folded state, bound to Doc, the N-terminal segment of Phd$^{52–73Se}$ is distinctly amphipathic with its hydrophobic side interacting with Doc. The side chains Leu-59, Phe-60, and Leu-63 of Phd$^{52–73Se}$ become completely buried on this hydrophobic surface, creating an extension of the hydrophobic core of Doc. Phe-56, which is more exposed, extends this set of interactions by packing against Leu-12 and Leu-81 of Doc (Fig. 4).

The C-terminal segment of Phd$^{52–73Se}$ is highly hydrophilic and provides only a single hydrophobic residue (Leu-70 in Phd, Se-Met-70 in Phd$^{52–73Se}$) to the binding interface. This residue makes extensive contacts with a small hydrophobic cavity on the Doc surface (Fig. 4B). The Phd-Doc contact surface in this region shows a high degree of charge complementarity with several negatively charged side chains of Phd$^{52–73Se}$ (Glu-55, Asp-61, and Asp-64) interacting favorably with positive residues in the Phd-binding groove of Doc$^{H66Y}$ (Arg-19 and Arg-85). Most striking in this region of Phd$^{52–73Se}$ are the interactions involving Asn-67. This residue is completely buried in the interface, its side chain protruding inside a small hydrophilic pocket where it makes complementary hydrogen bonds with the side chain of Asn-16 and Asn-78 of Doc (Fig. 4).

It should be noted here that although the C terminus of Phd$^{52–73Se}$ is adjacent to the surface cluster of conserved residues, the conserved sequence motif of Doc is not part of the Phd-binding site. This indicates that Phd$^{52–73Se}$ counteracts the toxic activity of Doc either by inducing a conformational change in Doc or by sterically preventing Doc to interact with the ribosome. Both mechanisms have been proposed earlier on for other TA modules (27, 28).

Doc Has an Incomplete Fic Fold—Structural similarity searches against the Structural Classification of Proteins (SCOP) data base using the DALI server failed to reveal any protein with significant similarity to Doc. However, all Doc homologues possess a conserved central motif (see above) of 9 residues that is shared with two other protein families: the bacterial cAMP-induced filamentation protein (Fic) and a domain of the eukaryotic Huntington Yeast Protein E (HYPE) protein (29). Sequence similarity between Doc and these other two protein families outside this 9-residue region is very weak with overall sequence identities below 15%.

A query of the Protein Data Bank with the conserved central motif of Doc resulted in the identification of two proteins that show a high structural similarity to Doc. These otherwise undescribed recent depositions from the Midwest Center for Structural Genomics are crystal structures of two Fic proteins: Fic_Hp from Helicobacter pylori (PDB entry 2F6S) and Fic_Nm from Neisseria meningitidis (PDB entry 2GO3). Fic_Nm is the most closely related to Doc with an root mean square deviation of 2.3 Å for 105 matching Ca atoms (Z score 6.7) (Fig. 5A). A structure-based sequence alignment is given in Fig. 5B.

Fic in most respects resembles the architecture of Doc, including very similar conformations for the conserved central motif in loop α3-α4, suggesting a common evolutionary origin.
His-13, the residue that anchors the conserved central motif, is conserved and makes equivalent interactions in all structures (supplemental Fig. S2). Nevertheless, Fic differs from Doc by the presence of an extra N-terminal helix, by an insertion in the loop 1-2, and most importantly, by an additional C-terminal helix (Fig. 5A). The latter adopts a position and conformation in the Fic structure that is strikingly similar to the position and conformation of the Phd peptide in the Doc-peptide complex (Fig. 5A). Removing Phd52–73Se from the complex unveils a large hydrophobic patch that extends toward the hydrophobic core. This is suggestive of an incomplete protein, and indeed, free Doc is poorly soluble and prone to aggregation and misfolding. Based upon these observations, we propose that Doc evolved from a Fic-like ancestor of which the C-terminal helix was transferred to a DNA-binding domain, thereby generating the antitoxin Phd. Upon binding, the antitoxin donates a helix to form the complex thereby complementing the fold.

**Doc Induces Growth Arrest but Not Cell Death**—Induction of Doc leads to growth arrest within the doubling time of *E. coli*. However, the cells do not lyse and remain motile for several hours after induction, when examined under a light microscope. This indicates an intact cell membrane and a working proton motive force. We do not observe filamentation in cells in which Doc has been activated, although filamentation but not induction of the SOS pathway was reported earlier (12). The observed growth arrest is reversible as cells replated in the absence of IPTG are capable of colony formation for several hours after the start of IPTG induction. We further confirmed that Doc arrests bulk protein synthesis but not RNA or DNA synthesis (Fig. 6). Additionally, we observed that Doc inhibits protein synthesis in a cell-free expression system and that Phd prevents this inhibition (data not shown).

**Doc Induces RelE-mediated Cleavage of Model mRNAs**—A recent study showed that Doc expression in *E. coli* strain
BL21(DE3) led to mRNA stabilization (13). By contrast, we observe destabilization of two different model mRNAs (lpp and dksA) after induction of doc in E. coli strain MG1655 (Fig. 7A and supplemental Fig. S3). Primer extension analysis after doc induction of lpp and dksA mRNAs reveals cleavage patterns very similar to that induced by RelE, especially just downstream of the start codons (Fig. 7B; supplemental Figs. S3 and S4). However, in contrast to RelE, Doc does not induce mRNA cleavages near the stop codons. Strikingly, non-translated versions of the lpp and dksA mRNAs (start codons changed to AAG) are not affected by induction of doc (Fig. 7A and supplemental Figs. S3 and S4). Thus, like RelE-induced cleavage, Doc-induced mRNA cleavage depends on translation. These results suggest that the cleavage is due to the activation of endogenous TA loci rather than a direct effect of Doc itself. Indeed, Doc-induced mRNA cleavage is not observed in a strain deficient in the five major E. coli TA modules (mazEF, chpBk, relBE, yoeB/yefM, and dinJ/yafQ-MG1655Δ5) (Fig. 7B, lanes 7–8). Moreover, expression of Doc in a strain that lacks relBE (MG1655ΔrelBE) also fails to induce Doc-mediated mRNA cleavage (Fig. 7B, lanes 11–12), indicating that ectopic production of Doc activates endogenous RelE. A previous report (30) indicated that Doc induces MazF activity. However, we did not observe mRNA cleavage at ACA sites, the signature sequence of MazF-mediated mRNA cleavage (31). Neither do we observe any influence of deleting mazEF on the cleavage pattern. Therefore our results do not confirm that Doc activates MazF. RelE has previously been described to be activated during nutritional stress due to Lon-mediated degradation of RelB antitoxin. The Doc-induced cleavage sites depend on Lon (Fig. 6B, lanes 9 and 10). These results are consistent with the proposal that the Doc-mediated inhibition of translation leads to RelE activation via Lon-dependent decay of RelB. Thus, the mRNA decay seen after induction of doc appears to be an indirect consequence of Lon-dependent activation of RelE. Consistent with this hypothesis is the observation by Liu et al. (13) that mRNA is not destabilized by induction of doc in E. coli strain BL21(DE3). BL21(DE3) is optimized for protein production and lacks the Lon and OmpT proteases (32).

DISCUSSION

The phd/doc locus of prophage P1 is an archetype member of a family of toxin-antitoxin modules found also on bacterial chromosomes. Although first reported in 1993 (12), phd/doc has remained less understood than the well known ccdAB, relBE and mazEF modules. Here we show that the toxin Doc adopts an all-α-helical fold different from the folds of other TA toxins with known structure. Doc contains a single highly conserved surface patch distinct from its interaction site with the antitoxin Phd. The significance of this conserved surface region is corroborated by the observation that Doc resembles members of another family of bacterial proteins called Fic, that a signature sequence present in the conserved surface region of Doc is also conserved.
in Fic; and that mutations within this motif affect Doc toxicity. This is indicative of a functionally relevant site and suggests that the mechanisms of action of Doc and Fic are related.

Doc has been shown to inhibit protein synthesis and to associate with the 70 S ribosome and with the 30 S ribosomal subunit (13). The mechanism of action of Fic is currently unknown. The strong structural similarities between both proteins and the presence of a highly conserved and functionally important region raise the possibility that Fic is also capable of halting translation on the ribosome or to modify ribosomal activity. Available data indicate that Fic has a role in cell division (33), most likely under the tight control of the cell division machinery. Mutations of Fic, such as G55R (34), at the center of the Phd-binding site on Doc are likely remnants of the N-terminal α-helix of Fic, which has no counterpart in Doc and is shown in purple. B, structure-based sequence alignment of Doc with Fic_Nm (PDB entry 2G03) and Fic_Hp (PDB entry 2F6S). The proteins were aligned to Doc using SALIGN (40). Residues with a structural match to Doc are shown in purple, and those that are structurally divergent from Doc and do not allow for a 1:1 match are shown in lowercase. The α-helices in Doc are indicated above the sequence. Residues corresponding to the conserved sequence motif in loop 3-α4 are highlighted in red, and conserved residues are indicated with asterisks. The loop α1-α2 is highlighted in blue. Residues from the C-terminal α-helix from the Fic proteins are shown in green, and those of the N-terminal α-helix are shown in purple.

During complex formation, Phd donates its C-terminal domain to complement the truncated Fic-like fold of Doc. Fold complementation has been described as one of the major mechanisms by which proteins can bind peptides in a β-strand conformation (35). As a result of the complex formation, the added β-strand complements for a missing secondary structure element in an otherwise incomplete fold. The completion of an Ig-like β-sandwich in the subunit-subunit and chaperone-subunit interactions in bacterial pili assembled by the chaperone-usher pathway and the addition of the hepatitis C virus NS4A cofactor peptide to the N-terminal β-sheet in the NS3 protease that complements a chymotrypsin-like fold are classical examples of this mechanism (36, 37). To our knowledge, this mechanism has not been observed before for α-helical peptide ligands binding to all-α-helical proteins. The amphipathic nature of the α-helix of Phd and the hydrophobic surface patch at the center of the Phd-binding site on Doc are likely remnants of the Fic-like ancestor of Doc, which had its hydrophobic core partially disrupted when it lost its C terminus.

Our in vivo experiments indicate that the ectopic overexpression of Doc can induce the mRNA interferase activity of RelE, a chromosomal TA toxin of E. coli. Activation of RelE, together with MazF, is triggered by stress conditions and is probably part of the general response of the cell to internal alarm signals. RelE activation may be an attempt of the cells to relieve the stress induced by Doc-mediated translation arrest. Indeed, RelE activity correlates with tmRNA activity (38). Thus,
mRNA degradation on Doc-arrested ribosomes may mimic translation quality control systems that are invoked when termination of translation fails.

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