The Intrinsically Disordered Domain of the Antitoxin Phd Chaperones the Toxin Doc against Irreversible Inactivation and Misfolding*

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Background: The doc/phd family is a paradigm for toxin-antitoxin modules in particular and protein-protein regulation in general.

Results: Without Phd and because of its incomplete Fic fold, Doc is heterogeneous and aggregation-prone and could dimerize in solution at high concentration.

Conclusion: Phd acts as an antiaggregation chaperone, protecting the toxin Doc from misfolding.

Significance: The intrinsically disordered domain of antitoxins can expand protein functionality.

The toxin Doc from the phd/doc toxin-antitoxin module targets the cellular translation machinery and is inhibited by its antitoxin partner Phd. Here we show that Phd also functions as a chaperone, keeping Doc in an active, correctly folded conformation. In the absence of Phd, Doc exists in a relatively expanded state that is prone to dimerization through domain swapping with its active site loop acting as hinge region. The domain-swapped dimer is not capable of arresting protein synthesis in vitro, whereas the Doc monomer is. Upon binding to Phd, Doc becomes more compact and is secured in its monomeric state with a neutralized active site.

Prokaryote genomes often contain one or more small operons consisting of a gene encoding for a toxic protein that interferes with the basic metabolism of the cell and a second gene that encodes a protein that neutralizes this toxin (1–4). The antitoxin gene is usually located upstream of the toxin gene and encodes a DNA binding domain involved in autoregulation of the operon. Commonly known as type II toxin-antitoxin modules, these gene pairs have been involved in the general stress responses of bacteria, and evidence is accumulating for a central role in the switch to the persister phenotype (5–7).

The phd/doc module on bacteriophage P1 represents one of the many distinct families of toxin-antitoxin modules found in bacterial chromosomes and plasmids (4). It contributes to stabilize P1 plasmid prophage in its plasmidic state in an Escherichia coli population (8) and is crucial for maintaining a high frequency of persistent cells in Salmonella (9). The toxin Doc is an inhibitor of translation related to Fic domain proteins (10–13). Doc is a kinase that catalyzes the phosphorylation of the translation elongation factor (EF)2-Tu (11). Binding of the C-terminal intrinsically disordered neutralization domain of the antitoxin Phd inhibits the activity of Doc (13, 14). In contrast, the N-terminal domain of Phd constitutes a DNA binding fold and acts as a transcription factor that self-regulates the operon (12, 14).

Regulation of transcription of the phd/doc operon occurs through a mechanism termed “conditional cooperativity” (12, 15–17). This entails that Phd on its own is only a poor repressor of the operon and requires the toxin Doc acting as co-repressor or co-activator for the efficient operon regulation (12, 18). At Doc to Phd ratios smaller or equal to 1, Doc enhances the affinity of Phd for its operator and significantly enhances repression. At higher Doc to Phd ratios, repression drops again, allowing transcription of the operon (12, 18). The molecular mechanism behind this conditional cooperativity was recently elucidated and entails a switch from a low to a high affinity interaction between toxin and antitoxin (12, 16, 17, 19).

Conditional cooperativity ensures that Doc and Phd are always in balance in the cell and that all Doc is sequestered in the form of Phd-Doc complexes of various stoichiometries. Activation of the system occurs through degradation of Phd by the ClpXP protease and leads to cell death after loss of the P1 plasmid (20).

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The abbreviations used are: EF, elongation factor; Ni-NTA, nickel-nitrilotriacetic acid; SEC, size exclusion chromatography; SAXS, small angle x-ray scattering; Rg, radius of gyration; CCS, collision cross-section; IM, ion mobility.
In this study, we further explored the role of Phd in regulating the activity of Doc. We show that Phd not only counteracts the toxic activity of Doc but also helps to keep Doc in a correctly folded monomeric state.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Wild-type Doc, Phd, and Doc\(^{H66Y}\)—**Cloning and expression of Doc, Phd, and Doc\(^{H66Y}\) from pET21b-docH66Y and pET21b-phd/doc in *E. coli* BL21(DE3) have been described (21). After growing the cells transformed with either pET21b-docH66Y or pET21b-phd/doc in LB medium at 310 K until the A\(_{600}\) was between 0.6 and 0.8, expression of the genes was induced by adding 1 mM isopropyl \(\beta\)-thiogalactosidase. The cells were harvested by centrifugation 2 h after induction and subsequently resuspended in 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.1 mg/ml 4-(2-aminomethyl)-benzenesulfonyl fluoride hydrochloride, 1 mg/ml leupeptin. The resuspended pellets were passed through a cell cracker at 277 K, and cell debris was removed by centrifugation. The clear extract was loaded onto a Ni-NTA affinity column pre-equilibrated in 20 mM Tris/HCl, pH 8.0 and washed with the same buffer to remove non-binding contaminants.

In the case of Doc\(^{H66Y}\), bound proteins were eluted with a linear 10-column volume gradient of imidazole (0.0–1.0 M) in 250 mM NaCl. Fractions containing Doc\(^{H66Y}\) were collected, concentrated, and loaded onto a Superdex 75 16/60 column (Amersham Biosciences) pre-equilibrated with 20 mM Tris/HCl, pH 8.0. Doc\(^{H66Y}\) eluted from this column in two peaks, which were pooled and used separately. To produce wild-type Doc and Phd, the procedure described by Garcia-Pino et al. (12) was followed.

**Analytical Gel Filtration**—Analytical size exclusion chromatography (SEC) experiments were performed in 20 mM Tris, pH 7.5, 150 mM NaCl on a Superdex 75 HR 10/30 column at a flow rate of 0.3 ml/min or a Shodex KW 402.5-4F HPLC column at a flow rate of 0.2 ml/min both at room temperature. Proteins were applied onto the column at a concentration of \(\approx\) 2.5 mg/ml in a volume of 200 or 20 \(\mu\)l. For preparing the complexes, the Phd(52–73) peptide was dissolved into solutions containing the different Doc species in a 1:1 ratio and incubated for 24, 96, and 144 h at 37 °C before the experiment. Commercial gel filtration standards (Bio-Rad) were used to calibrate the analytical gel filtration column.

**In Vitro Translation Assay**—The toxic activities of wild-type and mutant (H66Y) Doc monomer and dimer were tested in vitro by using the *E. coli*-based EasyXpress Protein Synthesis kit (Qiagen). Here, highly productive *E. coli* cell lysates containing all translational machinery components were used to express in vitro a reporter gene encoding the C-terminal His\(_6\) tag-labeled EF-Ts. If the toxin is active, translation is inhibited, and no EF-Ts product protein can be detected by Western blotting. Doc monomer and Doc dimer (both at 450 nM final concentration) were added to a mixture containing cell lysate, reaction buffer, and RNase-free water. The addition of the *ef-ts* DNA template started the in vitro synthesis. After 1 h of incubation at 37 °C, the reaction was stopped by adding 4 \(\times\) NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen). The samples were separated by 15% SDS-PAGE, and the reporter protein was detected by Western blotting. Purified wild-type Phd (500 nM) was used to show specific inhibition of Doc.

**Small Angle X-ray Scattering**—Small angle x-ray scattering data were collected at the SWING beamline of the SOLEIL synchrotron (Paris, France) in HPLC mode. The protein was loaded onto the SEC column (Shodex KW 402.5-4F) upstream of the small angle x-ray scattering (SAXS) capillary at 8 mg/ml in 50 mM HEPES buffer, 150 mM NaCl, pH 7.4 using a flow rate of 0.2 ml/min. Such a setup couples a size exclusion system to the SAXS data collection. The samples run through a SEC column and directly into the capillary where they are exposed to x-rays. This method allows the removal of aggregates or concentration-dependent issues, the collection of hundreds of scattering curves from the SEC peak that contains the sample of interest, and a nearly perfect match with the reference buffer (22).

The ATSAS package (23) was used to calculate the radius of gyration (\(R_g\)) of the different particles by Guinier analysis as well as the other SAXS-derived parameters listed in Table 3. For all samples, Guinier plots of the data show very good linearity, indicating the absence of aggregation. The indirect Fourier transform package GNOM (23) was used to compute the distance distribution \(P(r)\) functions from the scattering curve and calculate the maximum dimension of the particles (\(D_{max}\)). CRYOSOL (20) was used to compare the experimental data with the scattering curve computed from all the different models. *Ab initio* shape reconstruction based on the scattering data was done with the program DAMMIF from the ATSAS package (23).

To validate the quality of the SAXS-based models, we used the metrics recently proposed by Rambo and Tainer (24) that make use of the *s*(*l*(*s*)) versus *s* invariant. The \(\chi^2\) given by Equation 1 defines the weighted sum of the squared residuals between the model and data, whereas the \(R_{SAS}\) (Equation 2) incorporates residuals between modeled and experimental values for both \(R_g\) and \(V_c\) (Equations 3). In every case, the quality of our models is in excellent agreement with the experimental data, and the fitting parameters of the models to the data are within acceptable values (3) (see Table 3).

\[
\chi^2 = \sum_{i=1}^{N} \left[ \frac{P_{obs}(q_i) - P_{exp}(q_i)}{\sigma_i^2} \right]^2
\]

\[
R_{SAS} = \left( \frac{R_g^{exp} - R_g^{model}}{R_g^{exp}} \right)^2 + \left( \frac{V_c^{exp} - V_c^{model}}{V_c^{exp}} \right)^2
\]

\[
V_c = \frac{V_p}{2\pi l_c}
\]
SCALEPACK (25). Intensities were converted to structure factor amplitudes using TRUNCATE from the CCP4 program suite (26). The structure was determined by molecular replacement with PHASER (25) using Protein Data Bank code 3DD7 as a search model (13). The search identified the positions of eight molecules in the asymmetric unit. Refinement was done using phenix.refine (27) using a structure factor amplitude-based maximum likelihood target. Rounds of manual model building in Coot (28) were alternated with refinement of atomic positions and individual B factors by simulated annealing. Near the end of the refinement, simulated annealing was dropped, water molecules were added, and Translation/Libration/Screw (TLS) refinement was introduced. The statistics for data collection and refinement are given in Table 1.

Stability Calculations—All the structures used for the calculations (Protein Data bank codes 3DD7 (13), 3DD9 (this work), and 3K33 (12)) are refined at a resolution of 2.4 Å or better. Prior to the modeling, they were submitted to a short molecular dynamic simulation (100 ps) using GROMACS (29), and the calculations of the thermodynamic stability in terms of ΔG of unfolding were performed using the FoldX3.0 force field and tool suite (30). The results of the calculations are shown in Table 2.

Native Mass Spectrometry—Samples of wild-type Doc were prepared at 12 μM protein concentration in 10 mM ammonium acetate buffer, pH 6.9 and introduced into the vacuum of the mass spectrometer using nanoelectrospray ionization with in-house-prepared gold-coated glass capillaries with a voltage of +1.6 kV. Spectra were recorded on a quadrupole TOF instrument (Q-TOF2, Waters, Manchester, UK) modified for transmission of native, high m/z protein assemblies as described previously (31). Ion mobility spectra were recorded on a traveling wave ion mobility quadrupole TOF instrument (Synapt G2 HDMS, Waters) tuned to maintain native protein structure in the gas phase. Ion mobility data were calibrated against proteins of known structure as described elsewhere (32). Collision cross-sections (CCSs) for comparison of protein structures were derived from the coordinates of Doc monomer (Protein Data Bank code 3K33 or 3DD7), DocH66Y dimer (Protein Data Bank code 3DD9), the SAXS model of DocC (this work), and the structures calculated from the molecular dynamics simulations. All CCSs from Protein Data Bank files were calculated with the Mobical algorithm using the projection approximation (PA) method at 298 K in helium and scaled to the experimentally derived factor of 1.14 (PA) method at 298 K in helium and scaled to the experiments. All CCSs from Protein Data Bank files were calculated with the Mobical algorithm using the projection approximation (PA) method at 298 K in helium and scaled to the experimentally derived factor of 1.14 × CCS_PA × (MW_exp/MW_model)2/3 (33) to correct for the difference in ion mobility gas (N2, in ion mobility mass spectrometry (IM-MS) and helium in projection approximation calculations) and the fact that the projection approximation algorithm tends to underestimate cross-sections.

Molecular Dynamics Simulations—Molecular dynamics simulations for the different species were performed using the program GROMACS (29). In the case of the extended and partially folded Doc monomer, the coordinates of a DocH66Y subunit from the dimer crystal structure were used as starting point for the simulation. Missing loops and residues were modeled with Modeller. Prior to the molecular dynamics simulations, the molecules were prepared and energy-minimized using the program GROMACS (29) standard protocol. All hydrogen atoms were explicitly included. The proteins were embedded in a water box using a padding distance of 8 Å. Hydrogen atoms were constrained using the shake algorithm. The simulations were carried out at constant temperature (310 K) and constant pressure (1 atm) with periodic boundary conditions. 1000 frames were collected from the 10-ns simulations.

Isothermal Titration Calorimetry—All isothermal titration calorimetry titrations were carried out on an ITC200 apparatus (GE Healthcare). Prior to the measurement, DocH66Y and the C-terminal region of Phd (Phd(52–73)) were dialyzed to 150 mM NaCl, 100 mM sodium phosphate buffer, pH 7.0. The samples were filtered and degassed for 10 min before being examined in the calorimeter, and the titrations were performed at 30 °C. All the experiments consisted of injection of constant volumes of 2 μl of titrant into the cell (200 μl) with a stirring rate of 1000 rpm. Nominal sample concentrations were between 0.5 and 2 μM in the cell and 6 to 10 μM in the syringe. Actual sample concentrations were determined after dialysis by measurement of their absorption at 280 nm. All data were analyzed using the MicroCal Origin ITC 7.0 software using the 1:1 interaction model.

RESULTS

Doc Exists as a Mixture of Monomers and Dimers in Its Free State—Doc inhibits translation and is therefore toxic to E. coli. To produce wild-type Doc, co-expression of the antitoxin Phd is required. C-terminally His-tagged WT Doc can be obtained in its free form by loading the Phd-Doc complex on a Ni-NTA column and removing Phd via a step gradient of guanidinium hydrochloride. Doc then needs to be refolded, which results in a mixture of Doc monomers and dimers as judged by analytical size exclusion chromatography. Doc elutes from a Superdex 75 10/30 HR column as two peaks at 13.2 and 14.6 ml, corresponding to apparent molecular masses of 31.8 and 16.0 kDa, respectively, indicating an equilibrium between monomers and dimers (Fig. 1A). The monomer-dimer ratio can be modulated by the ionic strength used during the refolding conditions: low ionic strength will favor formation of the monomer, whereas high ionic strength will favor formation of the dimer.

As dimer formation via our unfolding/refolding protocol may be an artifact, we turned to a H66Y mutant of Doc that is known to be non-toxic to E. coli (18). C-terminally His-tagged DocH66Y can be expressed in the absence of Phd and purified using simple Ni-NTA affinity chromatography. The resulting preparation again elutes from a gel filtration column as two peaks, corresponding to a majority of dimer and a minority of monomer. Thus dimers of Doc are formed in E. coli in the absence of in vitro unfolding and refolding.

We used native IM-MS to assess the degree of heterogeneity present in the sample. IM-MS is rapidly gaining interest to assess both structural and dynamic aspects of proteins and complexes as the technique does not average across populations of species. Different conformational states of a protein can be separated in IM-MS even if they share similar charge and mass based on their gas-phase mobility when traversing a gas-filled cell. The time an ion spends in this cell is directly related to its rotationally averaged CCS and can thus be used as a struc-
tural restraint or to assess the degree of compaction of a protein.

IM-MS confirmed that in their free state both Doc and DocH66Y are highly heterogenic with respect to their conformational state. The protein solution consists of a population of folded monomers, dimers, and an extended monomeric state as shown in the drift time versus m/z contour plot. DocH66Y yielded a very similar mass spectrum compared with the wild type, indicating that the H66Y mutant is structurally similar to Doc. Blue and green dots represent monomeric and dimeric Doc species, respectively. Int, intensity.

**FIGURE 1. Dimerization precludes the action of Doc.** A, analytical SEC of Doc (in red), Doc bound to Phd(52–73) (in blue), and DocH66Y (in green). Molecular weight standards are colored in orange. The inset shows SDS-PAGE with the PageRuler Prestained Protein Ladder standards (15, 25, 35, 55, and 70 kDa from the bottom to the top) in lane 1, Doc in lane 2, monomer Doc in lane 3, and Doc bound to Phd(52–73) in lane 4. As expected, all the samples display the same pattern. B, Doc activity test monitored through the inhibition of the in vitro synthesis of EF-Ts (12). The concentration of Doc used in the assay was 450 nM. C, time-resolved inhibition of protein synthesis. Addition of Phd(52–73) to the reaction volume after 1 and 5 h of incubation with Doc does not rescue protein synthesis. By contrast, if Phd(52–73) is premixed with Doc (last five lanes) EF-Ts is synthesized. D, both monomeric DocH66Y and dimeric DocH66Y are inactive; the mutation as such is sufficient to relieve toxicity. Abs, absorbance; a.u., arbitrary units.

**FIGURE 2. Native MS analysis of Doc conformations.** A, native IM-MS of Doc: different conformational states of Doc were separated. IM-MS confirmed that in its free state Doc is highly heterogenic with respect to its conformational state. B, Doc in solution consists of a population of folded monomers, dimers, and an extended monomeric state as shown in the drift time versus m/z contour plot. C, DocH66Y yielded a very similar mass spectrum compared with the wild type, indicating that the H66Y mutant is structurally similar to Doc. Blue and green dots represent monomeric and dimeric Doc species, respectively. Int, intensity.

In contrast, the wild-type Doc dimer had no effect on the production of EF-Ts, indicating that the Doc dimer constitutes an inactive species (Fig. 1B). As the DocH66Y mutant is primarily produced in the dimer state, we next evaluated whether the reduced toxicity of this protein might be solely due to its tendency to dimerize. Both monomeric DocH66Y and dimeric DocH66Y were inactive in our in vitro translation assay, showing that the mutation as such is sufficient to relieve toxicity (Fig. 1D). Thus dimer formation and mutation are two independent routes toward the inactivation of Doc.
**TABLE 1**

X-ray data collection and refinement statistics
Parameters for the highest resolution shell are in parentheses. EMBL, European Molecular Biology Laboratory; r.m.s., root mean square.

| Parameter                          | DocH66Y dimer |
|-----------------------------------|---------------|
| Data set                          | DocH66Y      |
| Beamline                          | X13 (EMBL Hamburg) |
| Wavelength (Å)                    | 0.8081       |
| Space group                       | P2₁          |
| Unit cell                         |              |
| a (Å)                             | 53.1         |
| b (Å)                             | 198.0        |
| c (Å)                             | 54.1         |
| β (°)                             | 93.0         |
| Resolution limits (Å)             | 15.0–2.45 (2.51–2.45) |
| Number of measured reflections    | 149,678 (10,116) |
| Number of unique reflections      | 40,809 (3,915) |
| Completeness                      | 99.7 (100)   |
| Rmerge (%)                        | 0.10 (0.53)  |
| R factor (%)                      | 7.7 (2.5)    |
| R free factor (%)                 | 20.8 (23.8)  |
| R free factor (%)                 | 24.0 (27.5)  |
| Ramachandran profile              |              |
| Core                              | 96.1         |
| Other allowed                     | 3.3          |
| Outliers                          | 0.6          |
| r.m.s. deviations                 |              |
| Bond lengths (Å)                  | 0.01         |
| Bond angles (°)                   | 1.56         |
| Number of atoms                   |              |
| Protein                           | 6,856        |
| Water                             | 37           |
| Other                             |              |
| B factors (Å²)                    |              |
| From Wilson plot                  | 44.3         |
| All atoms                         | 52.2         |
| Protein atoms                     | 52.2         |
| Water atoms                       | 55.6         |
| Other atoms                       |              |
| Protein Data Bank code            | 3DD9         |

**Domain Swapping Stabilizes the Doc Dimer**—Crystals of the DocH66Y dimer contain eight molecules in their asymmetric unit. The structure was determined by molecular replacement and refined to R/R_free = 20.8%/24.0% with excellent stereochemistry (Table 1). The conformation of DocH66Y adopted in this crystal form is essentially identical to that of monomeric wild-type Doc in complex with Phd or the C-terminal domain thereof (12, 13) except that the protein folds open, creating two subdomains consisting of helices α1–α3 and α4–α6 and exposing the hydrophobic core (Fig. 3, A–D). Two such elongated monomers then associate to form a symmetric domain-swapped dimer involving a large (~2600-Å²) contact surface (Fig. 3, E and F). If swapping was ignored and a pseudomonomer consisting of residues 1–64 from one Doc molecule and residues 72–125 from the second Doc molecule was compared with the true monomeric Doc from the DocH66Y,Phd(52–73) complex, an overall root mean square deviation of 1.19 Å was obtained for all Ca atoms.

The hinge region involved in domain swapping consists of residues Gly⁶⁵–Ala⁷¹ (Fig. 3, C and D) and thus contains the catalytic loop of the Fic enzymes (10). In the monomeric state, a strained αCα conformation for Tyr⁶⁶ and Ala⁷¹ was observed (Fig. 4, A and B). This conformational strain was relieved in the domain-swapped conformation where both helices α2 and α4 also extend to cover these two residues. This discharge in conformational strain is likely a significant contributing factor to promote dimer formation. As a result of the swapping event, the loop containing residues Gly²¹–Ser²⁷ becomes highly flexible and is poorly defined (Fig. 3C). This contrasts with the conformation observed in the DocH66Y,Phd(52–73) complex. There this region is very well defined and adopts a rather extended conformation that makes extensive contacts with the active site loop (Gly⁶⁵–Asn⁷²) and likely helps to stabilize its strained conformation. In the domain-swapped dimer, this conformation for Gly²¹–Ser²⁷ is not possible because of steric reasons.

**Solution Structure of the Wild-type Doc Dimer**—In solution, wild-type Doc constitutes a heterogeneous conformational ensemble as observed by NMR (34) and native IM-MS (see above). We used SAXS to characterize the structure of the wild-type and H66Y mutant versions of Doc monomer and homodimer in solution (Fig. 5, A–D, and Table 2). Ab initio shape reconstruction of the wild-type Doc dimer using the program DAMMIF (23) showed that compared with the globular shape of the monomer (13) the envelope of Doc2 has a more elliptical cross-section and resembles a V-shaped particle as observed in the crystal structure of the DocH66Y dimer (Figs. 3E and 5F).

To obtain a pseudoatomic description of the wild-type Doc dimer, we started constructing our model from the coordinates of the x-ray structure of DocH66Y dimer (Protein Data Bank code 3DD9). The missing and mutated loops were modeled with Modeller (35), and a standard molecular dynamic simulation of 5 ns as implemented in GROMACS (29) was used to allow relieving the molecule from possible crystal lattice strains. We applied the same protocol to model the structure of the DocH66Y dimer in solution based on SAXS data collected in the same way as the wild type. The models reproduced the experimental scattering curve remarkably well (Fig. 5, A–D) and are highly compatible with the ab initio shapes reconstructed using DAMMIF (Fig. 5E; for details, see “Experimental Procedures”). They are also completely consistent with each other, indicating that both wild type and H66Y mutant form the same swap dimer in solution (Fig. 5F).

We further confirmed the validity of our model by comparing the collision cross-sections obtained experimentally from native IM-MS with those calculated for the structures based on the SAXS data. The experimental CCS values obtained for the Doc dimer were smaller than those calculated for the generated models. It has been suggested for IM-MS that large unstructured regions might not be stable in the gas phase and might collapse onto the structure. Removing these unstructured regions in the CCS calculations has yielded more accurate values compared with the CCSs obtained experimentally by IM-MS (36–38). Indeed, removing the last 11 amino acids from the C-tail of the Doc dimers (disordered in solution) yielded more accurate CCSs (Table 3). Moreover the analysis of the IM-MS data suggests that the dimer co-exists with monomeric forms, which explore conformations more elongated than observed for the compact wild-type Doc monomer. These conformations are compatible with a partially unstructured ensemble of Doc (Table 3). All this is consistent with the observed
poor stability of Doc and heterogeneous nature of the ensemble in the absence of Phd.

Phd Binding Leads to Formation of a Unique Complex—We next examined the interaction of monomeric and dimeric Doc with the neutralization domain of Phd (Phd(52–73)). Both monomeric Doc and dimeric Doc interact with Phd(52–73), but only a single species of complex is formed. This species formed from either Doc monomer or dimer elutes from a Superdex 75 10/30 HR SEC column with a higher retention time than the dimer and approximately the same retention time as the Doc monomer. This suggests that this species corresponds to the one previously crystallized (13) and consists of a Doc monomer around which a single Phd(52–73) is wrapped.

Small angle x-ray scattering confirmed these results. Table 2 shows the SAXS parameters obtained from the scattering curves of free monomeric Doc, Doc-Phd(52–73), and the Doc dimer. From the analysis of the SAXS parameters (Fig. 5, A, C, and G, and Table 2), it is clear that the observed species of wild-type Doc-Phd(52–73) agrees well with the aforementioned crystal structure of the DocH66Y-Phd(52–73) complex (13).

Phd Reverts the Doc Dimer to Its Monomeric Form—To assess whether Phd was capable of returning Doc to its monomeric form, we incubated pure Doc and DocH66Y dimers with equimolar amounts of Phd(52–73) at 37 °C and took samples at different times to characterize them by SEC and quantify the degree of dimerization. The analysis of the peaks corresponding to the monomeric and dimeric forms of both Doc and DocH66Y showed that after 24 h two-thirds of the dimeric forms were converted to monomer by Phd(52–73) with the process completed beyond 95% after 144 h (Fig. 6 A and 6 B).

We next monitored by isothermal titration calorimetry the binding of Phd(52–73) to the monomeric (Fig. 6 C) and dimeric (Fig. 6 D) forms of DocH66Y to assess the impact of
the dimer formation on the affinity for the neutralization domain of the antitoxin. The experiment showed that the DocH66Y monomer bound significantly tighter to Phd(52–73) with approximately a 150-fold drop in affinity upon dimerization. This suggests that upon Doc aggregation the antitoxin Phd would tightly bind to monomeric Doc (in equilibrium with the dimer), tilting the equilibrium against dimer formation (Fig. 7). Moreover even induced fit events could also be at play to facilitate the way to a fully bound antitoxin, which would disrupt the dimer interface.

**Phd Chaperones Doc against Inactivation and Misfolding**

FIGURE 4. **Conformationally dynamic regions of Doc.** The hinge region involved in domain swapping consists of residues Gly65–Ala71. This region contains the catalytic loop of the Fic enzymes numbered in the DocH66Y dimer (A) and in the structure of a compact Doc monomer (B). B also highlights the conformation of the loop Gly21–Ser27 in the monomeric conformation (as observed in Protein Data Bank codes 3DD7 and 3K33). This loop is missing in the x-ray structure of the DocH66Y dimer (A). Secondary structural elements are colored in green, and loop regions are in cyan.

FIGURE 5. **Low resolution structure of the domain-swapped wild-type and H66Y Doc dimers by SAXS.** Experimental SAXS curves of the wild-type Doc (A), wild-type Doc dimer (B), DocH66Y (C), wild-type DocH66Y dimer (D). E, ab initio envelope of Doc, (shown as a blue meshed surface) calculated from the experimental scattering data. Superimposed are the pseudoatomic coordinates of the minimal ensemble that best describes the scattering data of the wild-type Doc dimer. The model was constructed based on the crystal structure of the DocH66Y dimer and includes all missing residues (for further details, see “Experimental Procedures”). F, stereoview of the Co trace of the domain-swapped wild-type Doc (in blue) and DocH66Y (in red) dimers. Both dimers are consistent with each other with differences restricted to those regions that are not visible in the crystal structure, suggesting that both adopt the same conformation in solution. G, experimental SAXS curve of Doc bound to Phd(52–73). In all cases, experimental scattering curves are shown in gray, and the computed curves calculated from the pseudoatomic coordinates of each model are shown in black. a.u., arbitrary units.
Phd Chaperones Doc against Inactivation and Misfolding

TABLE 2
SAXS parameters
Theoretically and experimentally determined small angle X-ray scattering parameters of the different Doc species are shown. The quality of the SAXS-based models was assessed based on the metrics recently proposed by Rambo and Tainer (24). $\chi^2_{X_{free}}$ and $R_{SAX}$ which make use of the s(S)/s invariant (for further details, see “Experimental Procedures”). Theoretical and calculated values are shown in parentheses.

| Sample          | $R_g$     | $D_{max}$ | Molecular mass | $V_v$ | $\chi^2$ | $\chi^2_{X_{free}}$ | $R_{SAX}$ |
|-----------------|-----------|-----------|----------------|-------|----------|---------------------|-----------|
| Doc             | 16.9 (16.2) | 56.0      | 16.2 (14.7)    | 184 (181) | 0.53     | 0.48                | 0.0020    |
| Doc$^{H66Y}$    | 17.0 (16.2) | 55.5      | 13.3 (14.8)    | 197 (194)  | 1.90     | 2.40                | 0.0024    |
| Doc-Phd(52–73)  | 16.77 (16.82) | 58.0      | 17.6 (17.6)    | 191 (187)  | 0.40     | 0.57                | 0.0004    |
| Doc$^2$         | 23.7 (22.99) | 72.0      | 35.0 (29.4)    | 327 (309)  | 1.19     | 1.50                | 0.0039    |
| Doc$^{H66Y}_2$  | 23.7 (22.7) | 73.3      | 29.9 (29.6)    | 338 (355)  | 1.69     | 1.87                | 0.0056    |

TABLE 3
Mass spectrometry cross-section calculation
Collision cross-sections determined from the IM-MS data (Experimental CCS) and from atomic models based on x-ray diffraction and SAXS data and molecular dynamics simulations (Atomic model cross-section; for further details, see “Experimental Procedures”). Where multiple, closely related models are reported, the average CCSs with their standard deviations are given.

| Species                  | Experimental CCS | Atomic model cross-section |
|--------------------------|------------------|----------------------------|
|                          | $A^2$            | $A^2$                      |
| Doc                      | 1419 (6+ charge state) | 1435                       |
| Doc (partially unstructured monomer) | 169 (10+ charge state) | 1666 (6 models)            |
| Doc$^2$                  | 2289 (9+ charge state) | 2924 ± 54 (6 models)       |
|                          | 2341 ± 47 (6 models with C-terminal floppy tails removed) | |

Doc binds and stabilizes the DNA binding domain of Phd in what constitutes the basis for the conditional cooperativity phenomenon (12). Given that this binding also leads to the completion of the Fic fold of Doc, we expected that it would also stabilize the protein significantly. Indeed, when we used the FoldX force field to compare the thermodynamic stabilities of the bound and unbound forms of Doc, we observed a significant increase ($\Delta G = 11.1$ kcal/mol) upon binding to the Phd antitoxin domain (Table 4). Binding of a second Phd unit at an additional lower affinity site (12) involved only a slight increase in thermodynamic stability ($\Delta G = 3.8$ kcal/mol).

The dimerization via swapping of Doc$^{H66Y}$ led to a stabilization of the fold ($\Delta G = 10.0$ kcal/mol) comparable with that of the binding of the C-terminal domain of Phd, which was even higher in the case of the dimer of wild-type Doc ($\Delta G = 18.8$ kcal/mol). This suggests that the dimer will be favored in solution. Under physiological condition, P1-infected E. coli would always contain an excess of Phd, which is transcribed and translated before Doc. This is an intrinsic property of this operon that stems from its gene order and codon usage (8). Gel filtration showed that despite the dimer stability the Doc-Phd(52–73) complex remains stable in solution for over a week without any observable dimer formation. Therefore in all likelihood, once the Doc-Phd complex is formed in the cell, it will remain stable without inactivation by dimerization until the toxin is released by the proteolytic degradation of the antitoxin.

DISCUSSION

Regulation of toxin-antitoxin modules is known to occur at several levels: transcription, translation, protein activity, and protein lifetime. For F-plasmid ccd as well as for P1 phd/doc, these levels of regulation have been shown to be highly entangled with the intrinsically disordered nature of the toxin-neutralizing domain of the antitoxin being crucial for each of them (12, 39). Our present work suggests that in the phd/doc module the C-terminal domain of the antitoxin Phd is involved in an additional layer of regulation by protecting Doc against misfolding and domain swapping oligomerization.

Domain swapping is a common route of misfolding and aggregation (40). In some cases, domain swapping can result in gain of function. The examples of the polymerization of the pilus subunits (41), the activation of Bax (42), and the switch between the storage and assembly functions of the C-terminal domain of the spider silk protein (43) are but a few cases. Here the process of domain swapping is a simple consequence of the marginal stability of proteins, and oligomerization becomes a route to a lower free energy state. In eukaryotic organisms, these events often have severe consequences and are at the root of many aggregation-based diseases.

In prokaryotes, Doc functions as a kinase that tunes the cell pace by phosphorylating EF-Tu (11). Deletion of the phd/doc operon in Salmonella has a severe impact on the frequency of persistent cells upon macrophage infection (9), and bacteriophage P1 phd/doc is involved in one of the mechanisms at play to support plasmid maintenance in the host cell (8). The basis of Doc catalysis lies in the cleavage of a pyrophosphate bond-containing metabolite and transfer of a phosphate (or phosphate derivative) moiety to a target (10, 11). These events are mediated by a highly conserved active site motif (HXX/FX(D/E)(A/G)N(G/K)/R) common to all Fic enzymes (10), which in the case of Doc involves His$^{66\alpha}$, also at the heart of the dimerization event.

Domain swapping in Doc is likely linked to its incomplete Fic fold (13). The evolution of Doc from an AMPylase to a kinase involved the loss of an $\alpha$-helix compared with other Fic domains, and it has been suggested that this $\alpha$-helix was transferred to the C terminus of Phd during an illegitimate recombination event (10, 13). It is therefore not surprising that the Doc swap dimer is inactive as a kinase because the catalytic loop involving His$^{66\alpha}$ is disrupted as a consequence of dimerization.

This structural disruption also resulted in a reduced thermodynamic stability and possibly a higher degree of dynamics in the absence of its binding partner Phd (34). The domain-swapped Doc dimer compensates for this by the creation of a large dimer interface (2600 $Å^2$, which is almost 2.5-fold the contact area between Doc and Phd (12)). Binding of Phd or its C-terminal domain to Doc restores the Fic fold, resulting in a higher thermodynamic stability and reduced dynamics, and thus protects Doc against irreversible inactivation through dimerization (Fig. 7).
FIGURE 6. Phd(52–73) reverts the Doc and DocH66Y dimers to their monomeric forms. Shown is the time course analysis of the effect of Phd(52–73) on the stability of the wild-type (A) and H66Y (B) Doc dimer. Phd(52–73) was mixed in equimolar amounts with pure samples of both dimeric forms (in black) at 37 °C, and the amount of dimer was quantified by analytical SEC (for further details, see “Experimental Procedures”), taking samples from the reaction volume after 0 (i.e. pure dimer; in magenta), 24 (blue), 96 (green), and 144 h (orange). In both cases, the peak representing the pure monomeric forms is shown in dark cyan. The analysis of the peaks corresponding to the monomeric and dimeric species shows that the process is completed beyond 95% after 144 h. The dotted line shows the molecular weight (M.W.) standards used to calibrate the SEC. The interaction between DocH66Y monomer (C) and dimer (D) with the neutralization domain of Phd (Phd(52–73)) was monitored by isothermal titration calorimetry. Phd(52–73) and DocH66Y form a very tight complex (Kd/H11005 1.0 nM). Dimerization has a pronounced effect, decreasing the affinity to 144.0 nM. Abs, absorbance; a.u., arbitrary units.

FIGURE 7. Phd chaperones Doc against aggregation and misfolding. Given the pronounced difference in affinity for Phd between the monomeric and dimeric states of Doc, it is likely that Phd works as a chaperone by tilting the monomer-dimer equilibrium against dimer formation (1–3). Once bound to Doc (4), the Phd-Doc complex is very tight and stable; thus the toxin remains protected against aggregation and misfolding. Only the action of the proteolytic machinery can release the toxin. In the figure, Doc is colored in light blue and green, and the different conformational states are represented as in Fig. 3. The C-terminal neutralizing domain of Phd is shown as a bent cylinder colored in yellow.
TABLE 4

| Doc, - | Doc\textsubscript{Phd}, bound in the high affinity site | Doc with Phd, bound in the high and low affinity sites |
|--------|------------------------------------------------|------------------------------------------------|
| kcal/mol | 18.8 | 10.0 |

The highly crowded environment of the cell requires the action of chaperones to ensure the correct folding of many proteins. Some intrinsically disordered proteins are known to act as highly specific steric chaperones (44). Thus, a chaperone function of the intrinsically disordered segment of Phd and in extension possibly of other antitoxins as well provides advantages that ensure the proper functioning of the system. Indeed, at least two other toxin-antitoxin toxins, \textit{E. coli} RelE and YoeB, are known to dimerize (16, 45). These toxins are monomers in their active form and bind to the ribosome to transform it to degrade mRNA (45, 46). In this sense, one might draw a functional parallel between the antitoxins that protect toxins from misfolding and aggregation until they are released by proteolytic cleavage and the propeptides ofzymogens that protect proteases from degradation until they reach the appropriate activation site (47).

Moreover this chaperone function of the intrinsically disordered domain of Phd suggests that the N-terminal or C-terminal regulatory element found in the vast majority of Fic NMPylases occupying the position where Phd binds (10) could also have an additional function of preventing aggregation besides the regulatory action on the enzymes (10).

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REFERENCES

1. Buts, L., Lah, J., Dao-Thi, M. H., Wyns, L., and Loris, R. (2005) Toxin-antitoxin modules as bacterial metabolic stress managers. Trends Biochem. Sci. 30, 672–679
2. Gerdes, K., Christensen, S. K., and Løbner-Olesen, A. (2005) Prokaryotic toxin-antitoxin stress response loci. Nat. Rev. Microbiol. 3, 371–382
3. Hayes, F. (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301, 1496–1499
4. Yamaguchi, Y., Park, J. H., and Inouye, M. (2011) Toxin-antitoxin systems in bacteria and archaea. Annu. Rev. Genet. 45, 61–79
5. Gerdes, K., and Maisonneuve, E. (2012) Bacterial persistence and toxin-antitoxin stress response loci. Annu. Rev. Microbiol. 66, 103–123
6. Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol. Lett. 230, 13–18
7. Tripathi, A., Dewan, P. C., Barua, B., and Varadarajan, R. (2012) Additional role for the ccd operon of F-plasmid as a transmissible persistence factor. Proc. Natl. Acad. Sci. U.S.A. 109, 12497–12502
8. Lehnherr, H., Maguin, E., Jaffé, S., and Yarmolinsky, M. B. (1993) Plasmid addiction genes of bacteriophage P1: doc, which causes cell death on curing of prophage, and phd, which prevents host death when prophage is retained. J. Mol. Biol. 233, 414–428
9. Helaine, S., Cheverton, A. M., Watson, K. G., Faure, I. M., Matthews, S. A., and Holden, D. W. (2014) Internalization of \textit{Salmonella} by macrophages induces formation of nonreplicating persisters. Science 343, 204–208
10. Garcia-Pino, A., Zenkin, N., and Loris, R. (2014) The many faces of Fic structural and functional aspects of Fic enzymes. Trends Biochem. Sci. 39, 121–129
11. Castro-Roa, D., García-Pino, A., De Gietter, S., van Nuland, N. A., Loris, R., and Zenkin, N. (2013) The Fic protein Doc uses an inverted substrate to phosphorylate and inactivate EF-Tu. Nat. Chem. Biol. 9, 811–817
12. Garcia-Pino, A., Balasubramanian, S., Wyns, L., Gazit, E., De Greve, H., Magnuson, R. D., Charlier, D., van Nuland, N. A., and Loris, R. (2010) Allostery and intrinsic disorder mediate transcription regulation by conditional cooperativity. Cell 142, 101–111
13. Garcia-Pino, A., Christensen-Dalsgaard, M., Wyns, L., Yarmolinsky, M., Magnuson, R. D., Gerdes, K., and Loris, R. (2008) Doc of prophage P1 is inhibited by its antitoxin partner Phd through fold complementation. J. Biol. Chem. 283, 30821–30827
14. Smith, J. A., and Magnuson, R. D. (2004) Modular organization of the Phd repressor/antitoxin protein. J. Bacteriol. 186, 2692–2698
15. Loris, R., and Garcia-Pino, A. (2014) Disorder- and dynamics-based regulatory mechanisms in toxin-antitoxin modules. Chem. Rev. 114, 6933–6947
16. Bøggild, A., Sofos, N., Andersen, K. R., Feddersen, A., Easter, A. D., Passmore, L. A., and Brodersen, D. E. (2012) The crystal structure of the intact \textit{E. coli} RelE toxin-antitoxin complex provides the structural basis for conditional cooperativity. Structure 20, 1641–1648
17. Overgaard, M., Borch, J., Jørgensen, M. G., and Gerdes, K. (2008) Messenger RNA interferase RelE controls relBE transcription by conditional cooperativity. Mol. Microbiol. 69, 841–857
18. Magnuson, R., and Yarmolinsky, M. B. (1998) Coexpression of the P1 addiction operon by Phd and Doc. J. Bacteriol. 180, 6342–6351
19. Winther, K. S., and Gerdes, K. (2012) Regulation of enteric \textit{vap}BC transcription: induction by \textit{VapC} toxin dimer-breaking. Nucleic Acids Res. 40, 4347–4357
20. Svergun, D. I., Barberato, C., and Koch, M. H. (1995) CRYSOIL—a program to evaluate x-ray solution scattering of biological macromolecules from atomic coordinates. J. Appl. Crystallogr. 28, 768–773
21. Garcia-Pino, A., Dao-Thi, M. H., Gazit, E., Magnuson, R. D., Wyns, L., and Loris, R. (2008) Crystalization of Doc and the Phd-Doc toxin-antitoxin complex. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 64, 1034–1038
22. David, G., and Perez, J. (2009) Combined sampler robot and high-performance liquid chromatography: a fully automated system for biological small-angle x-ray scattering experiments at the Synchrotron SOLEIL SWING beamline. J. Appl. Crystallogr. 42, 892–900
23. Konarev, P. V., Petoukhov, M. V., Volkov, V. V., and Svergun, D. I. (2006) ATSAS 2.1, a program package for small-angle scattering data analysis. J. Appl. Crystallogr. 39, 277–286
24. Rambo, R. P., and Tainer, J. A. (2013) Accurate assessment of mass, models and resolution by small-angle scattering. Nature 496, 477–481
25. McCoy, A. J., Grosse-Kunstleve, R. W., Storoni, L. C., and Read, R. J. (2005) Phenix refinement framework. Acta Crystallogr. D Biol. Crystallogr. 61, 458–464
26. Collaborative Computational Project, Number 4 (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. 50, 760–763
27. Afonine, P. V., Grosse-Kunstele, R. W., and Adams, P. D. (2005) The Phenix refinement framework. CCP4 Newsletter 42
28. Emalsky, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501
29. Lindahl, E., Hess, B., and van der Spoel, D. (2001) GROMACS 3.0: a package for molecular simulation and trajectory analysis. J. Mol. Model. 7, 306–317
30. Van Durme, J., Delgado, J., Stricher, F., Serrano, L., Schymkowitz, J., and Rousseau, F. (2011) A graphical interface for the FoldX forcefield. Bioinformatics 27, 1711–1712
31. Sobott, F., Hernández, H., McCammon, M. G., Tito, M. A., and Robinson, C. V. (2002) A tandem mass spectrometer for improved transmission and analysis of large macromolecular assemblies. Anal. Chem. 74, 1402–1407
32. Bush, M. F., Hall, Z., Giles, K., Hoyes, J., Robinson, C. V., and Ruotolo, B. T.
(2010) Collision cross sections of proteins and their complexes: a calibration framework and database for gas-phase structural biology. Anal. Chem. 82, 9557–9565

33. Hall, Z., Politis, A., Bush, M. F., Smith, L. J., and Robinson, C. V. (2012) Charge-state dependent compaction and dissociation of protein complexes: insights from ion mobility and molecular dynamics. J. Am. Chem. Soc. 134, 3429–3438

34. De Gieter, S., Loris, R., van Nuland, N. A., and Garcia-Pino, A. (2014) 1H, 13C, and 15N backbone and side-chain chemical shift assignment of the toxin Doc in the unbound state. Biomol. NMR Assign. 8, 145–148

35. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., and Sali, A. (2006) Comparative protein structure modeling using Modeller. Curr. Protoc. Bioinformatics Chapter 5, Unit 5.6

36. Pagel, K., Natan, E., Hall, Z., Fersht, A. R., and Robinson, C. V. (2013) Intrinsically disordered p53 and its complexes populate compact conformations in the gas phase. Angew. Chem. Int. Ed Engl. 52, 361–365

37. Jurneczko, E., Cruickshank, F., Porrini, M., Clarke, D. J., Campuzano, I. D., Morris, M., Nikolova, P. V., and Barran, P. E. (2013) Probing the conformational diversity of cancer-associated mutations in p53 with ion-mobility mass spectrometry. Angew. Chem. Int. Ed Engl. 52, 4370–4374

38. Atmanene, C., Petiot-Bécard, S., Zeyer, D., Van Dorsselaer, A., Vivat Hannah, V., and Sanglier-Cianférani, S. (2012) Exploring key parameters to detect subtle ligand-induced protein conformational changes using traveling wave ion mobility mass spectrometry. Anal. Chem. 84, 4703–4710

39. De Jonge, N., Garcia-Pino, A., Buts, L., Haesaerts, S., Charlier, D., Zangger, K., Wyns, L., De Greve, H., and Loris, R. (2009) Rejuvenation of CcdB-poisoned gyrase by an intrinsically disordered protein domain. Mol. Cell 35, 154–163

40. Gronenborn, A. M. (2009) Protein acrobatics in pairs—dimerization via domain swapping. Curr. Opin. Struct. Biol. 19, 39–49

41. Sauer, F. G., Füuterer, K., Pinkner, J. S., Dodson, K. W., Hultgren, S. J., and Waksman, G. (1999) Structural basis of chaperone function and pilus biogenesis. Science 285, 1058–1061

42. Czabotar, P. E., Westphal, D., Dewson, G., Ma, S., Hockings, C., Fairlie, W. D., Lee, E. F., Yao, S., Robin, A. Y., Smith, B. J., Huang, D. C., Kluck, R. M., Adams, J. M., and Colman, P. M. (2013) Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. Cell 152, 519–531

43. Hagn, F., Eisoldt, L., Hardy, J. G., Vendrely, C., Coles, M., Scheibel, T., and Kessler, H. (2010) A conserved spider silk domain acts as a molecular switch that controls fibre assembly. Nature 465, 239–242

44. Pauwels, K., Lustig, A., Wyns, L., Tommassen, J., Savvides, S. N., and Van Gelder, P. (2006) Structure of a membrane-based steric chaperone in complex with its lipase substrate. Nat. Struct. Mol. Biol. 13, 374–375

45. Kamada, K., and Hanaoka, F. (2005) Conformational change in the catalytic site of the ribonuclease YoeB toxin by YefM antitoxin. Mol. Cell 19, 497–509

46. Neubauer, C., Gao, Y.-G., Andersen, K. R., Dunham, C. M., Kelley, A. C., Hentschel, J., Gerdes, K., Ramakrishnan, V., and Brodersen, D. E. (2009) The structural basis for mRNA recognition and cleavage by the ribosome-dependent endonuclease RelE. Cell 139, 1084–1095

47. Stroud, R. M., Kossiakoff, A. A., and Chambers, J. L. (1977) Mechanisms of zymogen activation. Annu. Rev. Biophys. Bioeng. 6, 177–193