The toxin (Doc) and antidote (Phd) proteins of the plasmid addiction system of bacteriophage P1 were purified as a complex. Cocrystals of the complex contained a 2:1 molar ratio of Phd:Doc as assayed by dye binding following SDS-polyacrylamide gel electrophoresis and as determined by amino acid analysis. Gel filtration and analytical ultracentrifugation revealed that the two protein species interact in solution to form a P2D trimer composed of one Doc and two Phd molecules. These results support a model in which Phd inhibits the toxic activity of Doc by direct binding. Circular dichroism experiments showed that changes in secondary structure accompany formation of the heterotrimeric complex, raising the possibility that Phd may act by an allosteric mechanism. Studies of Phd and Doc molecules labeled with fluorescent energy donor and acceptor groups gave an equilibrium dissociation constant of about 0.8 μM and a very short, sub second half-life of complex dissociation. As a consequence, low concentrations of free Doc toxin are likely to be present both transiently and in the steady state in cells containing the Phd antidote, making mechanisms of single-hit Doc toxicity improbable.

The plasmid addiction system of bacteriophage P1 consists of two proteins: a toxin known as Doc (death on cure) (1). Bacteriophage P1 lysogenizes Escherichia coli as a low copy plasmid that is inherited in a remarkably stable fashion, in part, because the plasmid addiction system kills cells that have lost the P1 genome (1–5). The mechanism of Doc toxicity is unknown. Phd is, however, known to be degraded in a fashion dependent on the host ClpXP protease system, and continual synthesis of Phd is required to counteract Doc toxicity (3). In daughter host ClpXP protease systems, and continual synthesis of Phd is therefore required to counteract Doc toxicity (3). In daughter cells that have lost the P1 genome, degradation of Phd eventually results in reduction of antidote activity to a level where the host is killed. Phd may exert its own toxicity by binding Doc and physically blocking its interaction with cellular target molecules (1) or by acting in an allosteric fashion to alter Doc structure and toxin activity. Alternatively, Phd might exert its antidote activity indirectly by activating another protein that neutralizes Doc.

Some of the regulatory and biochemical properties of Phd have been established. For example, Phd is a DNA-binding protein that represses transcription of its own gene as well as that of Doc by binding to a operator DNA site that overlaps the addiction promoter (4). Four molecules of Phd bind to the intact operator with dimers binding to adjacent 10-base pair sub sites (6). Phd has a predominantly α-helical structure when bound to DNA or at low temperatures but the free monomeric protein has a Tm of 25 °C and is largely denatured at 37 °C (6).

Addiction systems involving a stable toxin and proteolytically unstable antidote are also used by other low copy plasmids and may be involved in the regulation of programmed cell death in E. coli (7–13). Although direct interaction between the toxin and its antidote is assumed to be the mechanism of inhibition for each of these systems, experimental evidence for complex formation has been presented only for the CsdA antidote and CcdB toxin of the F plasmid of E. coli (14). In the study of the phage P1 addiction proteins presented here, we demonstrate that Phd binds directly to Doc, forming a trimeric complex (P2D) with one molecule of Doc and two molecules of Phd. Complex formation appears to be accompanied by changes in secondary structure. P2D complexes form at low micromolar concentrations and are very short lived, ensuring a dynamic equilibrium between bound and free forms within the cell.
Hewlett Packard 8452A diode array spectrophotometer with extinction coefficients per tyrosine of 1,934 \text{ M}^{-1} \text{ cm}^{-1} (274 nm at pH 7) and 2377 \text{ M}^{-1} \text{ cm}^{-1} (294 nm in 0.1 \text{ M} KOH for Phd (1 Tyr) and Doc (4 Tyr)). Circular dichroism spectra were obtained using an AVIV 60DS spectropolarimeter equipped with a temperature-controlled sample holder and a 10-mm pathlength cuvette. Thermal denaturation experiments were performed in a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1 mM EDTA; samples were equilibrated at 1 °C temperature intervals for 1 min, and the ellipticity at 222 nm was averaged for 2 min.

**Ultracentrifugation and Gel Filtration—** Analytical ultracentrifugation experiments were performed using a Beckman Optima XL-A centrifuge. Solutions containing Phd and Doc from dissolved crystals were centrifuged overnight at 15,000 rpm. After reaching equilibrium (established by unchanged A_{474} readings at 1 h intervals), absorbance profiles were determined at 274 nm, and five scans were averaged for analysis. Data from experiments in which ln(A) versus r² plots showed curvature were fitted by nonlinear least-squares methods to a 2F + D ⇔ P,D equilibrium model using Eq. 1.

**Gel filtration experiments were performed by chromatographing 200-μl samples on a 10 × 300 mm Superdex 75 HR 10/30 column (separation range 3–70 kDa) using a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm, and the column was calibrated using gel filtration molecular weight standards (Bio-Rad).**

**RESULTS**

**Copurification of Phd and Doc—** As a first step in studying potential interactions between Phd and Doc, both proteins were purified from *E. coli* strain X90/pRDM032, a cell expressing the phage P1 addiction proteins under Ptac promoter control (4). As shown in Fig. 1A, Doc protein and the major portion of the Phd protein coeluted during ion-exchange chromatography on a MonoS column; a smaller quantity of Phd alone also eluted at a lower salt concentration than the Phd:Doc complex. During purification from cells without Doc, Phd eluted only in the low salt position. Further purification by reverse-phase HPLC chromatography under denaturing conditions separated Doc from Phd and resulted in purification of each protein to greater than 95% homogeneity (Fig. 1B). The identity of each protein was confirmed by MALDI-TOF mass spectrometry (Fig. 2). The observed masses (8,130 Da for Phd; 13,578 Da for Doc) are
Doc and Phd Form a Heterotrimeric Complex

**Fig. 3. Crystals of Phd-Doc complex.** A, photograph of crystals; B, SDS-polyacrylamide gel electrophoresis of washed and dissolved crystals shows presence of both Phd and Doc; C, denaturation of the Coomassie Blue-stained polyacrylamide gel is most consistent with a 2:1 ratio of Phd:Doc.

within 1 or 2 Da of those expected from the protein sequences, indicating that the formyl group of the N-terminal fMet residue is removed post-translationally for each protein.

**Phd and Doc Form a 2:1 Complex in Crystals—Solutions** containing high concentrations of purified Phd and Doc formed needle-shaped crystals that grew to a length of several millimeters after 24–48 h at 4 °C (Fig. 3A). Following washing, SDS-polyacrylamide gel electrophoresis showed that individual crystals contained both Phd and Doc (Fig. 3B). Densitometry of the Coomassie Blue-stained gel indicated that the Phd and Doc proteins were present in the crystal in a 2:1 molar ratio (Fig. 3A). Amino acid analysis of protein from washed crystals also supported a 2:1 molar ratio of Phd:Doc in the crystals (Table I). Hence, in the crystalline state, Phd and Doc appear to form a macromolecular complex containing twice as many Phd molecules as Doc molecules. The smallest oligomer consistent with this stoichiometry is a P2D trimer, but P2D3 hexamers, P2D4 nonamers, and Doc are also consistent with these data.

**Oligomeric State in Solution—** In gel filtration chromatography on Superdex 75, purified Doc eluted at an approximate volume expected for a monomer (data not shown); Phd had been previously shown to be monomeric in solution (6). To determine the solution form of Phd-Doc complexes, protein from dissolved cocrystals was also chromatographed on Superdex 75. At high loading concentrations (80 μM in Doc monomer equivalents), a major peak of approximately 30 kDa was observed (Fig. 4A). This value is consistent with formation of a P2D solution trimer (expected molecular mass, 29.8 kDa). Some trailing asymmetry was evident in the Fig. 4A column profile, suggesting that a small fraction of P2D complexes dissociated during chromatography. Indeed, when the sample was diluted 10-fold immediately before chromatography (loading concentration 8 μM in Doc monomer equivalents), less of the P2D peak was present and more prominent, lower molecular weight peaks corresponding to Doc and Phd alone were observed (data not shown). These experiments suggest that Phd-Doc complexes dissociate in the micromolar concentration range with kinetics on the time scale of minutes or faster. Gel filtration of a mixture of Phd:Doc in a 4:1 ratio revealed no species larger than 30 kDa but showed significant free Phd (data not shown). These results show that the P2D complex is the major solution form, even in the presence of excess Phd.

As a second assay of solution oligomeric form, protein from dissolved Phd-Doc cocrystals (80 μM in Doc equivalents) was analyzed by analytical ultracentrifugation. These data were fit best by a 2:1 ratio of Phd:Doc and showed significant deviations from the behavior expected for 1:1 or 2:4 complexes of Phd:Doc (Fig. 4B). Equilibrium centrifugation experiments performed using lower protein concentrations (loading concentration of 20 μM in Doc equivalents) showed curvature in the ln([A]273)/versus r2 plot, indicative of some complex dissociation. Fitting of this data to a 2P + D ⇄ P2D model gave an equilibrium dissociation constant of 1.0 ± 0.3 μM at 20 °C (Fig. 4C).

**Changes in Secondary Structure Induced by Complex Formation—** The circular dichroism (CD) spectrum of purified Doc had features expected for a protein containing approximately 50% α-helix (Fig. 5A). Melting experiments in a neutral buffer at 60 °C (Fig. 5C) showed that Doc is monomeric. The native structure of Doc (Tm = 60 °C) is quite stable in comparison to Phd (Tm = 25 °C) (6). In fact, Phd is almost completely denatured at 37 °C, whereas Doc is native at this temperature.

By comparing the CD spectra of a mixture of 2 μM Phd and 1 μM Doc with the summed spectra of the uncomplexed proteins, secondary structural changes induced by complex formation were assessed. At 4 °C, where Phd and Doc by themselves are both native, the mixture had slightly less ellipticity than the isolated proteins, suggesting that complex formation leads to some change in structure (Fig. 6A). At 37 °C, where Phd by itself is primarily unfolded, the P2D complex had greater ellipticity than the individual proteins, suggesting an increase in secondary structure upon binding (Fig. 6B). In the latter instance, it seems probable that at least a portion of Phd folds as it binds to Doc. Fitting of titration experiments, monitored by changes in CD ellipticity at 37 °C, gave an equilibrium dissociation constant of 0.7 ± 0.1 μM (Fig. 6C).

**Affinity and Kinetics of Complex Formation—** To study the affinity of complex formation by another method, we performed fluorescence resonance energy transfer experiments by titrating increasing amounts of rhodamine-labeled Doc (the acceptor molecule) against a constant amount of fluorescein-labeled Phd.
Because energy transfer depends on the inverse sixth power of the distance between the fluorescent dyes, any change in fluorescence should arise from interactions between the proteins. Fig. 7A shows fluorescence spectra, and Fig. 7B shows the change in fluorescence at 25 °C as a function of Phd concentration. These experimental data were fit best by a binding curve calculated for a model

\[ 2P + D \rightleftharpoons P_2D \quad K_d = [P]^2[D]/[P_2D] \quad (\text{Eq. 2}) \]

that is second-order in Phd and first-order in Doc, with an equilibrium dissociation constant of 0.83 ± 0.02 μM

To determine the half-life of complex dissociation, fluorescently labeled P_2D complexes were mixed with excess unlabeled Phd in a stopped-flow instrument, and the change in fluorescence was monitored (Fig. 7C). The rate constant of this exchange process, which should be limited by dissociation was 42 (± 7) s⁻¹ indicating that complex dissociation must occur very rapidly. Similar results were obtained when the dissociation reaction was initiated by mixing fluorescently labeled P_2D complexes with unlabeled Doc. The rapid dissociation rate is consistent with the tailing seen in gel filtration. From the equilibrium constant and a dissociation rate constant, a third-order association rate constant of approximately 6 \times 10^{13} M^{-2} s^{-1} can be calculated.

DISCUSSION

Efficient inhibition of the toxic activity of Doc by the Phd antidote is essential for maintaining the viability of bacteriophage P1 lysogens of E. coli (1) but little concrete information concerning the molecular mechanism of this inhibition has been available. The results presented here show that Phd and Doc interact directly to form a heterotrimeric complex in solution. In the absence of more detailed information, it is not possible to know whether formation of the Phd-Doc complex blocks the toxic activity of Doc by sterically occluding interactions with its cellular target or if formation of the complex alters the structure of Doc in a way that prevents this interaction. It is intriguing, however, that formation of the Phd-Doc complex appears to result in a small change in secondary structure. Were this alteration of structure to involve a portion of Doc, then this could serve as the mechanism of toxin neutralization.

The Phd-Doc complex is a P_2D heterotrimer. Heterotrimers
are not common in biological systems and must be assembled asymmetrically. A well known example with a 2:1 ratio of components is the heterotrimer of growth hormone with two extracellular receptor domains, in which each receptor binds to a distinct surface of growth hormone (16). The oligomeric form of the antidote-toxin complex for the ccd system is hexameric with two dimers of the toxin and one dimer of the antidote (14), but this architecture also requires asymmetric construction and, indeed, can be considered as a heterotrimer of dimers. We find it intriguing that there are 2:1 or 1:2 ratios of antidote:toxin in both the P1 addiction system and F ccd system. Whether these and other addiction systems share common structural features is not known.

Because simultaneous three-body collisions between protein monomers of Phd and Doc is not a statistically viable mechanism of assembly, formation of the P2D complex must proceed either through a P2 or a PD intermediate. Neither of these dimeric species has been detected in solution, implying that if either species can form then it must be unstable and poorly populated at equilibrium. We note, however, that two monomers of Phd bind cooperatively to operator subsites, suggesting that Phd subunits interact with each other in the DNA-bound state (6). It is also worth noting that the P2D solution complex may also be an active operator binding species; using a mutant form of Doc, Magnuson and Yarmolinsky (5) have suggested that a 2:1 ratio of Phd:Doc might occur in operator complexes.

The P2D complex of Phd and Doc is unstable by comparison with many macromolecular complexes. K_d values for complex dissociation of 0.7–1.0 μM^2 were obtained from analytical ultracentrifugation at 20 °C, from fluorescence resonance energy transfer at 25 °C, and from changes in CD ellipticity at 37 °C. Dissociation of unlabeled Phd–Doc complexes to free components was also observed in the micromolar concentration range by gel filtration. P2D complexes equilibrate with free subunits on a subsecond time scale in vitro, which should ensure rapid equilibration of the bound and free intracellular pools of Phd and Doc.

There is an approximate 3-fold ratio of Phd to Doc in overproducing cells. Because the phd and doc genes are part of the same operon, a similar ratio would be expected in P1 lysogens. Moreover, because Phd represses its own synthesis and that of Doc (1–5), P1 lysogens are unlikely to contain very high concentrations of either protein. To ensure 90–99.9% binding of Doc with a K_d of 0.7–1.0 μM^2, we calculate that the free Phd concentration would need to be from 2.2 to 36 μM (~2,200 to 36,000 molecules/cell). However, under these conditions there would still be free Doc concentrations of 260–22 nM. Hence, it is important to view the Phd-Doc interaction as buffering the free concentration of Doc rather than eliminating free Doc from

![Fig. 6. Changes in CD ellipticity accompany complex formation. A, CD spectra, at 4 °C, of 1 μM Doc and 2 μM Phd before and after mixing in a tandem cuvette; B, mixing experiment at 37 °C. Other conditions identical to panel A. C, increasing quantities of Doc were added to 2 μM Phd protein and the change in CD ellipticity at 222 nm was monitored. The solid line is calculated for the reaction 2P + D ⇌ P2D with a K_d of 0.72 μM^2. The buffer for all experiments was 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1 mM EDTA.](image)

![Fig. 7. Resonance energy transfer. A, fluorescence spectra of rhodamine-labeled Phd (0.8 μM), fluorescein-labeled Doc (0.02 μM), and a mixture of both proteins; B, complex formation assayed by resonance energy transfer. The solid line is calculated for the reaction 2P + D ⇌ P2D with a K_d of 0.81 μM^2. C, kinetics of dissociation of complexes containing rhodamine-labeled Phd and fluorescein-labeled Doc monitored by fluorescence after addition of a 250-fold excess of unlabeled Phd. The solid line is a single exponential fit with a rate constant of 42 s^-1. Experiments in all panels were performed at 25 °C in a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA.](image)
the cell. If the assumptions that underlie these rough calculations are sound, then free Doc at submicromolar levels must be relatively innocuous to cells. Otherwise, P1 lysogens would have a significant growth disadvantage relative to nonlyso- gens. However, higher levels of free Doc (presumably in the micromolar range) must be lethal for the plasmid addiction mechanism to function. These considerations make it extremely unlikely that the mechanism of Doc toxicity involves single hit enzymatic lethality and suggest that Doc may reversibly inhibit some required cellular activity in a cooperative fashion that results in a steep concentration dependence of toxicity.

In P1 lysogens, Phd binding spares the cell from Doc-mediated killing (3). However, Doc binding induces structure in denatured Phd at physiological temperatures, and thus it seems likely that Doc binding would also help protect Phd from degradation. Degradation of Phd is mediated directly or indirectly by the ClpXP protease (3), which consists of ClpP protease subunits and regulatory ClpX ATPase subunits (17–19). ClpX is responsible for substrate recognition by ClpXP and has been shown to recognize specific peptide sequences at the C termini of several substrate proteins (20–22). If ClpXP directly degrades Phd, then C-terminal substrate recognition is probably also involved. By this model, formation of the P2D complex with Doc could prevent degradation of Phd by sequestering C-terminal sequences required for ClpX recognition and perhaps by stabilizing at least some parts of Phd in a native structure. We note, however, that the rapid dissociation rate and relatively high equilibrium dissociation constant for P2D complexes makes it likely that a pool of free Phd, in a largely denatured form, is present even when Doc is present in P1 lysogens. If Phd were too good a substrate for ClpXP, then degradation of the free pool and mass action to produce more free Phd could lead to Doc toxicity even in lysogens. Hence, it seems likely that Phd should be a rather poor substrate for ClpXP. Indeed, in ClpXP<sup>−</sup> strains, Phd is degraded relatively slowly over the course of several cell generations (3).

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