The ccd addiction system plays a crucial role in the stable maintenance of the Escherichia coli F plasmid. It codes for a stable toxin (CcdB) and a less stable antidote (CcdA). Both are expressed at low levels during normal cell growth. Upon plasmid loss, CcdB outlivess CcdA and kills the cell by poisoning gyrase. The interactions between CcdB, CcdA, and its promoter DNA were analyzed. In solution, the CcdA-CcdB interaction is complex, leading to various complexes with different stoichiometry. CcdA has two binding sites for CcdB and vice versa, permitting soluble hexamer formation but also causing precipitation, especially at CcdA:CcdB ratios close to one. CcdA alone, but not CcdB, binds to promoter DNA with high on and off rates. The presence of CcdB enhances the affinity and the specificity of CcdA-DNA binding and results in a stable CcdA-CcdB-DNA complex with a CcdA:CcdB ratio of one. This (CcdA₂CcdB)ₙ complex has multiple DNA-binding sites and spirals around the 120-bp promoter region.

The 95-kb low copy number F plasmid is maintained in Escherichia coli with remarkable stability. Many synergistic processes are responsible for its maintenance in the bacterial population. The plasmid contains a partitioning system to distribute plasmid copies to the daughter cells during cell division as well as several site-specific recombination systems to resolve oligomeric plasmid molecules. In addition, the F plasmid and other low copy number plasmids encode programmed cell death systems: daughter cells, which did not inherit the plasmid, are killed. Such systems are called post-segregational killing or addiction systems (reviewed in Ref. 1). The F plasmid encodes three such systems: srm (stable RNA degradation) (1, 2), flm (F leading maintenance) (1, 3), and ccd (controlled cell death) (1, 4, 5).

The ccd system was the first one to be identified (6) and remains the best studied. The ccd operon encodes a toxin (CcdB; 101 amino acids, 11.7 kDa) and its antidote (CcdA; 72 amino acids, 8.3 kDa). The synthesis of the ccd proteins is autoregulated at the level of transcription by a complex of both toxin and antitoxin (7–9). Both proteins are expressed, the toxic activity of CcdB being reversibly inactivated by the presence of CcdA. The stability (10) as well as in vivo life span (11) of CcdB is higher than that of CcdA. It was postulated by us that the thermodynamic stability of CcdA is low enough to keep the protein close to unfolding in vivo conditions, whereby it facilitates its metabolism (10). Upon plasmid loss, CcdA is quickly degraded by the Lon protease (12, 13), leaving CcdB free to kill the cell. CcdB acts as a poison and inhibitor of DNA gyrase, an essential enzyme that catalyzes negative supercoiling of DNA (14–16). CcdA inhibits the lethal action of CcdB by directly binding the toxin (inactivation) and by the extraction of the toxin from its complex with the target gyrase (rejuvenation) (17, 18). The crystal structures of CcdB as well as that of a gyrase fragment have been solved and a model for the CcdB-gyrase complex proposed (19–21).

Still, crucial mechanistic aspects of the ccd system have remained unrevealed. Even some basic parameters such as the stoichiometry and binding constants of the intermolecular interactions involved are unknown. In the present paper, we investigate in detail the interactions between CcdA, CcdB, and specific operator DNA using a range of biophysical and biochemical techniques.

EXPERIMENTAL PROCEDURES

General—The purification of CcdA and CcdB was carried out as described before in (19). Electrospray mass spectrometry was carried out in a Quattro II quadrupole mass spectrometer (Micromass, Manchester, UK) having a m/z range of 4000, equipped with an electrospray interface as described previously (22).

Spectropolarimetry—The binding between CcdA and CcdB was monitored in near-UV CD in the spectral range from 280 to 295 nm. The protein solutions were centrifuged and filtered (0.45 μm) to remove turbidity. Approximately 4 ml (exact volume determined using analytical balances) of ~10 μM CcdB (concentration determined photometrically using the extinction coefficients from Dao-Thi et al. (10)) in the corresponding buffer (either 50 mM citrate, pH 5.6, 100 mM NaCl, or 50 mM cacodylate, pH 6.5, 100 mM NaCl) was placed in a thermostated cuvette with 1-cm optical path length and then titrated with ~60 μl of ~35 μM CcdA (exact concentration determined photometrically prior to titration) solution in the same buffer so that a molar ratio CcdB:CcdA of 2 was reached after around 10 additions. The progress of reaction was best monitored by the intensity of the negative peak at around 283 nm.

Chromatography—HPLC1 chromatography was carried out on a 600S Controller coupled to a 996 FDA detector (Waters, Milford, MA) equipped with a Rheodyne 9125 (Cotati, CA) injector using a reverse phase C4 column (4.6 × 25 mm) (214TP54) (Vydac, Hesperia, CA) equilibrated in 15% acetonitrile, 0.1% trifluoroacetic acid at 1 ml/min. The column was developed with a 50-min linear gradient from 10 to 50% acetonitrile at room temperature. Absorption data collection at 280
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nm was performed under Millennium (Waters). The column was calibrated with 1:1 and 1:2 mixtures of CcdA:CcdB. Peak heights were found to be the most accurate to calculate CcdA:CcdB ratios and were used as such.

For the size-exclusion experiments the CcdA:CcdB 1:2 complex was prepared by adding dropwise CcdA to CcdB in different buffer solutions to achieve a final concentration of 5 and 10 μM, respectively. The 500-μl mixture was incubated for 10 min at room temperature prior to injection (450 μl) on a Superdex75 HR 10/30 size-exclusion column (Amersham Biosciences, Inc., Uppsala, Sweden). The buffer solutions are: 50 mM sodium citrate, pH 5.0, 50 mM sodium cacodylate, pH 6.0, 50 mM Mops, pH 7.0, 50 mM Tris, pH 8.0, and 50 mM Bicine, pH 9.0. The Superdex75 HR column was, respectively, equilibrated in 50 mM buffer solution containing 50 mM KCl, 0.1 mM EDTA, and calibrated with a gel filtration standard from Bio-Rad, i.e. γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.35 kDa). All runs were performed at room temperature on an Akta-Explorer (Amersham Biosciences, Inc.).

For the DNA complex binding experiments: the Superdex75 HR column was equilibrated with 50 mM Tris, pH 8.0, 150 mM KCl, 0.1 mM EDTA. The CcdA:CcdB 1:1 complex was prepared by adding dropwise CcdA to the mixture of CcdB in 50 mM Tris, pH 8.0, to achieve a final concentration of 15 μM each. The CcdA:CcdB-DNA complex was prepared by adding dropwise CcdA to the mixture of CcdB and the 85-bp MH12 DNA fragment (see Fig. 5C) in 50 mM Tris, pH 8.0, to achieve a final concentration of 15 μM CcdA, 15 μM CcdB, and 80 μg/ml DNA or 5 μM CcdA, 10 μM CcdB, and 80 μg/ml DNA. In another experiment the CcdA₂CcdB₂ complex was formed, followed by a 10-min incubation with 60 μg/ml 65-bp MH12 DNA fragment in 50 mM Tris, pH 8.0. Each mixture (500 μl) was incubated for 10 min at room temperature prior to injection (450 μl). The procedure of preparing the mixtures is critical and has to be followed accurately to avoid possible precipitation.

Gel Retardation Assays and DNAse I Footprinting—Binding of CcdA and CcdB proteins to specific 5'-end-labeled DNA fragments was determined according to the method described by (25) with modifications. Protein-DNA complexes were formed in 20 μl of binding buffer (10 mM Tris-HCl, pH 7.4, 250 mM KCl, 5 mM MgCl₂, 2.5 mM CaCl₂, 0.5 mM dithiothreitol, 2.5% glycerol) in the presence of 0.1 μg of sonicated herring sperm DNA for 20 min at 37 °C. Samples were loaded on preelectrophoresed 6% (w/v) polyacrylamide gels in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). Electrophoresis was performed in the same TBE running buffer at room temperature at 8 V/cm for 3 h.

DNAse I footprinting was performed according to the method described by Galas and Schmitz (24). Purified proteins were incubated with 5'-end-labeled DNA fragments in 100 μl of binding buffer (see above, gel retardation) and further treated as described previously (25).

Isothermal Titration Calorimetry—Binding studies with CcdA and CcdB were carried out with a MicroCal™ Omega isothermal titration calorimeter. The concentration of the samples in the cell and the syringe was determined spectrophotometrically. Both proteins were dialyzed prior to titration against the same buffer using Spectra/Por™ CE (molecular weight cut-off, 5000; sample volume, 2 ml) at room temperature (cold room temperature causes precipitation of CcdA at high concentration) for 3 h. The titrations were carried out at a temperature of 25 °C.

Due to the complexity of the interaction, the standard software could not be applied meaningfully. The results were interpreted based on the inflection point and the shape of the thermograms. Semiquantitative conclusions on the strength of the involved microscopic interactions were obtained supposing that in the early stage of a titration, one type of interaction dominates the overall reaction.

Differential scanning calorimetry thermograms were simulated for different values of ρ = KCtot, where K is the binding constant, Ctot the macromolecular concentration in the cell before starting the titration, and n is the number of binding sites. Only for ρ values of around 5–50 sigmoidal curves are obtained. For values around unity and below, the titration curve is featureless. For ρ values of 100 or above, an abrupt transition (“box car”) is observed. From the shape of the experimentally measured thermograms, the parameter p is about 2000 for the forward titration (CcdA into CcdB) and 20 for the reverse titration (CcdB into CcdA). This leads to the following limits for the binding constants involved: 10⁴ M⁻¹ < K₁ < K₂ < 10⁷ M⁻³.

Numerical Simulations—The following model, where B₂ represents the CcdB dimer and A₂ the CcdA dimer, was set up.

\[\begin{align*}
K_{\text{H}} & \quad A_2 B_2 + A_1 \rightarrow A_1 B_2 A_2 \\
K_{\text{L}} & \quad A_1 B_2 + A_2 \rightarrow A_2 B_2 A_1 \\
K_{\text{H}} & \quad A_2 B_2 A_1 + A_1 \rightarrow A_2 B_2 A_2 \\
K_{\text{L}} & \quad A_2 B_2 A_1 + A_2 \rightarrow A_2 B_2 A_2 \\
K_{\text{H}} & \quad B_2 A_2 B_1 + A_1 \rightarrow A_2 B_2 A_1 \\
K_{\text{L}} & \quad B_2 A_2 B_1 + A_2 \rightarrow A_2 B_2 A_2 \\
K_{\text{H}} & \quad A_2 B_2 A_1 + A_2 \rightarrow A_2 B_2 A_2 \\
K_{\text{L}} & \quad A_2 B_2 A_1 + A_1 \rightarrow A_2 B_2 A_2 \\
\end{align*}\]

MODEL 1

Higher aggregates than A₂B₂A₂B₂ and B₂A₂B₂A₂ were neglected. These aggregates could already be considered to have the tendency to precipitate. Combining the above expressions for the equilibrium constants and the following mass balances.

\[\begin{align*}
A_{2,\text{total}} & = [A_2] + K_1[A_1][B_1] + K_2 K_0 [A_1][B_2]^2 + 2 K_0 K_2 [A_1]^2 [B_2] \\
& + 2 K_0 K_2 [A_2]^2 [B_2]^2 + 3 K_0 K_2 [A_1][B_2][B_2] + 2 K_0 K_1 K_2 [A_1][B_2][B_2] \\
& \quad \ldots \text{(Eq. 1)} \\
B_{2,\text{total}} & = [B_2] + K_1 [A_1][B_1] + K_0 [A_1][B_2] + 2 K_0 K_2 [A_1][B_2]^2 \\
& + 2 K_0 K_2 [A_2]^2 [B_2]^2 + 3 K_0 K_2 [A_1][B_2][B_2] + 2 K_0 K_1 K_2 [A_1][B_2][B_2] \\
& \quad \ldots \text{(Eq. 2)}
\end{align*}\]

The equilibrium concentrations were solved numerically using the Euler method. Iterations were carried out to minimize the difference between the calculated A₂,total and B₂,total and the actual values. The robustness of the model was checked by applying different binding constants within the same order of magnitude, giving essentially the same results. Two series of simulations were carried out. In the first one the total concentration of CcdB (B₂,total) was kept constant at 10⁻⁵ M dimer equivalents (0.47 mg/ml), and the total CcdA concentration (A₂,total) was varied from the thousandth to the thousandfold. In a second series of simulation the CcdA concentration was restrained and the amount of CcdB altered in an analogous way.

RESULTS

CcdA and CcdB Form More than One Type of Complex—

CcdA is thought to counteract the lethal effect of CcdB by forming a non-equivalent complex that prevents CcdB to interact with gyrase (17, 18). The stoichiometry of this complex is still not strictly defined (8, 17, 26), and both CcdA₁CcdB₂ and CcdA₂CcdB₁ have been suggested (16, 28). We have therefore used high resolution gel filtration chromatography experiments to observe such complexes and to determine experimental conditions suitable for a detailed characterization of their properties. CcdA:CcdB 1:2 and 1:1 mixtures were prepared and
analyzed on an analytical gel-permeation column Superdex75 HR at different pH values (pH 5 to pH 9).

For the 1:2 mixture, three major populations were observed (Fig. 1): a 65.5-kDa peak (at an elution volume of 10 ml) that is in agreement with a CcdA<sub>2</sub>CcdB<sub>4</sub> complex, a 24.4-kDa (elution volume 12 ml) peak corresponding to the dimer of CcdB, and a peak around 18-ml elution volume that contained aggregated and degraded CcdA and CcdB. The 16.6-kDa peak of the dimer of CcdA was only observed at pH 5.

The 65.5-kDa peak was analyzed on a C4-reverse phase column (C4-RPC), and its chromatographic profile at 280 nm is shown on the inset in Fig. 1. The peaks obtained from the reverse phase step were analyzed by electrospray mass spectrometry. CcdA elutes in two peaks with retention times of 39.4<sub>H1</sub> and 39.8<sub>H1</sub>, both with identical masses of 8372 Da, while CcdB elutes with a retention time of 48.6<sub>H1</sub> with a mass of 11704 Da. The masses determined with electrospray mass spectrometry match the calculated masses of CcdA and CcdB.

More than One Binding Constant Is Involved—CcdA has a flat and featureless CD spectrum in the region from 260 to 300 nm, allowing the titration of CcdA into CcdB to be followed in the near-UV CD spectrum of CcdB. As can be seen in Fig. 2A, two peaks between 280 and 300 nm shift in intensity as well as position (from 288.9 to 292.2 nm and from 282.2 to 283.8 nm). Fig. 2B shows the change in intensity of these two peaks when titrating CcdA into CcdB. A plateau is reached around a CcdA:CcdB ratio of 2. Around this ratio, addition of CcdA also causes local clouding. At a CcdA:CcdB ratio of 3 a persistent turbidity evolves. This coincides with a sagging of the intensity of the CD spectra. Similar, when adding CcdB to a CcdA solution, turbidity evolves at a very early stage.

Furthermore isothermal titration calorimetry (ITC) was used to study the interaction between CcdA and CcdB. ITC has
the advantage that it does not depend on the optical properties of a system but records a physical property inherent to almost all binding processes, the production or absorption of heat. Hence titrations can be carried out in both directions, i.e. having CcdB in the cell and adding small amounts of a concentrated CcdA solution as well as vice versa. For simple binding phenomena, both experiments are expected to produce the same results.

Interestingly, different starting points lead to different apparent stoichiometries and affinities (Fig. 3, A and B). Starting from an excess of CcdB, saturation is reached at a ratio CcdA:CcdB of 1:2. In the reverse case, starting from an excess of CcdA, a binding signal was recorded until a ratio of above 1:1 was reached. In both cases, but especially noticeably in the second condition that lead to an approximately 1:1 stoichiometry, the contents of the calorimetric cell was slightly turbid after the experiment. This is indicative of the formation of insoluble aggregates.

The two titration thermograms show a second difference: in the first case saturation is attained rather abruptly, whereas in the second case it is reached smoothly. This indicates that the interaction is characterized by more than one binding constant and/or might be partially irreversible.

A thermogram obtained from such a complex interaction cannot be analyzed with the standard software to obtain the involved microscopic binding constants. Rough estimates of the binding constants can be obtained if we assume that in the early phase of the transition (when one of the reactants is in excess) one binding constant dominates the process. Assuming a model with two microscopic binding constants $K_L$ and $K_H$, one constant will dominate the early stages of the forward titration, while the other will dominate in the early stages of the reverse titration. Such a model is described in detail in the following paragraph.

**CcdA-CcdB Interaction in a Cooperative Model** —To understand the complex behavior observed for the interaction between CcdA and CcdB, we performed numerical simulations of the forward and reverse titrations. We assumed a model in which CcdA and CcdB can form long chains in a cooperative way. Such a model is realistic given the strong tendency of CcdA and CcdB for forming precipitates when mixed in a 1:1 molar ratio and the possibility of producing a soluble CcdA$_2$CcdB$_4$ complex. In this cooperative interaction model, initial binding of a single dimer of CcdB$_2$ to CcdA$_2$ occurs with a binding constant $K_L$. Addition of a second molecule of CcdB$_2$ to an existing CcdA$_2$CcdB$_2$ complex involves a higher affinity constant $K_H$. In a similar way, higher molecular weight species are produced by addition of more CcdA$_2$ and CcdB$_2$ dimers using the same binding constants. The equilibrium equations of the model are given in the experimental procedures section. This cooperative model assumes a conformational change on the part of CcdA. Most likely, CcdA is partly unfolded when not bound to CcdB. Binding of a first CcdB$_2$ dimer to CcdA$_2$ results in proper folding and creates a more stable binding site for the second CcdB$_2$ dimer.

It is not possible to obtain correct values of $K_L$ and $K_H$ from the ITC experiments. However, the applied protein concentrations and the shape of the observed titration thermograms...
permit us to make a reasonable estimate for the range of the binding constants involved \(10^5 \text{M}^{-1} < K_L < 10^8 \text{M}^{-1}\) (29). Indeed a sigmoidal titration thermogram is only observed within this range. Smaller values result in a soft featureless increase, while larger values lead to an abrupt jump (Fig. 3C).

Based on the above considerations we estimated two numerical values for the binding constants: \(K_L = 10^6 \text{M}^{-1}\) and \(K_H = 5 \times 10^7 \text{M}^{-1}\).

Typical results of our simulations are given in Fig. 4. We calculated the distribution of the protein into different complexes mimicking the experimental conditions of the ITC titration experiments (relatively high concentrations: \(2 \times 10^{-5} \text{M}\) protein). Because of the robustness of the model the result is only marginally influenced by variations of the binding constants in the same order of magnitude. The calculation shows that at equimolar amounts of CcdA and CcdB most of CcdA is present in the form of the higher aggregates of the type \((A_2B_2)_n\). Only at a molar ratio CcdB:CcdA of above \(-3:1\), the soluble hexamer CcdA_4CcdB_2 will be the dominant form of CcdA. In Fig. 4B it can be seen that at this excess a maximal fraction of CcdB will also be in this hexameric form. Above this molar ratio most of CcdB will be in the form of the free dimer, because all the available CcdA is consumed in complexes. On the other hand at close to equimolar concentrations and at molar ratios CcdB:CcdA below 1, especially between 0.1 and 1, CcdB is found in higher aggregates and the hexamer CcdA_4CcdB_2. Such a situation can be characterized as an aggregation scenario, confirmed by gel filtration experiments.

The CcdA-CcdB Complex Spirals along the DNA—It is known from literature that both ccd proteins participate in the autoregulation of the system (7–9). Only few details on the interaction of the ccd proteins with the ccd operator DNA are known. CcdA binds on DNA and CcdB does not (also see below). To better characterize the binding site(s) of CcdA and the role

\[\text{Fig. 4. Simulation of the interaction of CcdA and CcdB in vitro. For a total concentration of CcdB of } 2 \times 10^{-5} \text{M and varying concentrations of CcdA semi-logarithmic diagram of: A, fractions of the different species in the total amount of CcdA versus the molar ratio CcdB:CcdA (for instance fraction } \langle A_2B_2A_2A_2 \rangle \text{ of } B_{\text{total}} = 2 \langle A_2B_2A_2A_2 \rangle / B_{\text{total}}; B, fractions of the different species in the total amount of CcdB versus the molar ratio CcdB:CcdA.}\]
of CcdB, we examined the DNase I footprint to a 157-bp fragment (F4R1; fragment from start of F4 to end of R1; see Fig. 5C). These experiments revealed a large region of interaction (~110 bp; Fig. 5, A and B), in agreement with previous data obtained with the lysate of a strain overexpressing the ccd proteins (9). Whereas Tam and Kline (9) only detected protection of one strand, we definitely observed a complex footprint for each strand. Protected stretches of ~7–10 nucleotides long are separated by 3–6-nucleotide-long segments that either remained normally accessible to the nuclease or became hyper-reactive to DNase I cleavage in the presence of both Ccd proteins. These alternating patches of protection and hypersensitivity toward digestion are mostly staggered by a few nucleotides toward the 3’-end on one strand with respect to the complementary partner (Fig. 5C). Such a pattern is consistent with a series of CcdA-CcdB complexes that spiral along a 120-bp region.

Footprinting experiments were performed using different CcdA-CcdB ratios ranging from 1:1 to 3:1. The observed footprint patterns are indistinguishable (Fig. 5A). In the absence of CcdB, only a slight effect but in no way a clear pattern of protection was observed (Fig. 5B). Mobility-shift experiments performed with an aliquot of the very same samples (data not shown) clearly demonstrated binding of CcdA to the operator fragment. The lack of a distinct footprint most likely reflects the formation of unstable CcdA-operator DNA complexes with high on and off rates (see below).

The Presence of CcdB Increases the Affinity of CcdA for DNA Binding—The region protected by the CcdA-CcdB complex against DNase I cleavage contains two interesting stretches, the promoter region, and a 6-bp palindromic sequence just downstream of the −10 promoter element (Fig. 5C). To better characterize the sequence requirements for binding of the Ccd proteins and to determine the minimal target site for CcdA-CcdB binding, we have performed gel retardation experiments with a variety of DNA fragments: the 157-bp F4R1 fragment and three subfragments thereof (OP12, Prom, and Pal; for a definition of these fragments, see Fig. 5C) that were synthesized according to the nucleotide sequence described by Tam and Kline (9).

Gel shift assays indicated that CcdA retards all four of these fragments, but CcdB does not. Fig. 6A shows the titration of F4R1 with CcdA. The transition from unbound to CcdA-bound F4R1 DNA is very sharp, pointing to a strong cooperative interaction.

Relatively low concentrations of CcdB (around 0.5 μM) are sufficient to provide an apparent increase in affinity of CcdA for F4R1. A larger shift of the DNA fragment is observed. The affinity of CcdA for F4R1 in the presence of CcdB is estimated to be about 10 times higher than that observed in its absence as measured by the amount of CcdA necessary to produce a band shift. Similarly, we demonstrated binding of CcdA alone and the CcdB-induced increase in the apparent binding constant and “supershifting,” for the 34-bp-long OP12 fragment (Fig. 6C). In contrast, binding of CcdA on the 25-bp fragment containing the palindrome sequence (Pal) was only detectable in the presence of CcdB, whereas binding on the 21-bp-long promoter sequence (Prom) was hardly detectable (Fig. 6C). Combined (see also DNase I footprinting), these results indicate that the palindrome region might constitute the nucleation site for binding of multiple CcdA-CcdB molecules to the control region of the ccd operon.

These retardation experiments confirm the results already obtained by Afif et al. (30).

CcdA Does Not Only Bind to the ccd Control Region—CcdA-CcdB binding was also tested for a 40-bp fragment bearing the target site of the Phd/Doc proteins of the bacteriophage P1 addiction system (31, 32) and for a 150-bp fragment carrying the promoter/operator region of the bipolar ArgECBFGH operon of a psychrophilic Mortierl strain (33) (Fig. 7A). In both

**Fig. 5.** DNase I footprinting. Autoradiographs of DNase I footprinting experiments of CcdA and CcdA plus CcdB to the 157-bp fragment F4R1. C → T and A → G are the corresponding Maxam-Gilbert sequencing ladders. Yellow bars indicate regions of protection against DNase I cleavage. A, footprinting of CcdA-CcdB at different concentrations and ratios (as indicated in μM); upper strand labeled. B, binding of CcdA (2.5 μM) and of equimolar concentrations of CcdA and CcdB (2.5 μM) to the F4R1 fragment (lower strand labeled). C, DNA sequence of the control region of the ccd operon. Sequences corresponding to the F4 and R1 oligonucleotides are underlined; regions corresponding to the OP12, Pal, Prom, and MH12 fragments are indicated by colored lines. The ATG initiation codon of CcdA is indicated in red. Yellow boxed areas correspond to sequences protected against DNase I digestion by CcdA-CcdB binding. The HindIII and HinfI restriction sites are indicated, and the palindrome is boxed in gray.
cases, a clear shift was observed in the presence of CcdA and a further shift when both CcdA and CcdB are present. In conjunction with the negative binding results to other DNA fragments (see below), these data suggest that CcdA-CcdB has a binding preference for promoter regions in general.

The Presence of CcdB Influences the Specificity of CcdA for DNA Binding—CcdA and CcdA-CcdB binding experiments were performed with a mixture of fragments of variable size obtained by a double HindIII/HinfI digestion of a pUC18 plasmid derivative harboring the ccd control region, revealed by EtBr staining. The 189-bp fragment carrying the control region is indicated with an arrow.
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Whereas other fragments of a size smaller than, similar to, or even larger than that of the specific target were not. In the presence of CcdB, less CcdA was required to obtain a complete shifting of the 189-bp fragment. In contrast, binding to the high molecular weight fragments was strongly reduced (Fig. 7B). It seems thus that not only the affinity but also the specificity of CcdA toward DNA is much lower in the absence of CcdB.

**The CcdA–CcdB–DNA Complex Is Stable—**CcdA–CcdB–operator complexes proved to be particularly stable. The addition of a 1000-fold excess nonlabeled F,R fragment to preformed CcdA–CcdB–operator complexes (at 0.88 μM each) resulted in an immediate slight increase in the migration velocity and dissociation of a small fraction of the complexes (less than 10%) (Fig. 6D). No further dissociation was detected even after 1-h incubation. The initial increase is probably due to the immediate dissociation of aspecifically and weakly bound CcdA molecules, while the CcdA–CcdB-bound complexes are very resistant to the specific competitor. Similar experiments with CcdA alone indicated a very short half-life of the complexes (data not shown). However, this experiment is difficult to perform in correct conditions as precipitation occurs at high DNA concentration and at the CcdA concentrations required to observe sufficient binding.

**CcdA and CcdB Bind to DNA in a 1:1 Ratio—**To further characterize the way CcdA and CcdB associate when bound to DNA, HPLC and size-exclusion chromatography experiments were carried out on the 85-bp DNA fragment MH12. This fragment is an extension of the fragment Pal used in the mobility shift assays (for an exact definition of this fragment, see Fig. 5C). The elution position of MH12 in presence of a 1:1 CcdA:CcdB mixture was evaluated on a Superdex75 HR 10/30 column equilibrated in 50 mM Tris, pH 8.0, 150 mM KCl, 0.1 mM EDTA (Fig. 8). The DNA elution peak (black curve) was shifted toward the void volume of the column in the presence of a CcdA:CcdB 1:1 mixture (red curve). This DNA peak shift was not observed when a CcdA:CcdB 1:2 (of the same CcdA concentration) mixture was added to the 85-bp DNA fragment MH12 or when CcdA was added dropwise to the DNA and CcdB mixture to obtain a final ratio CcdA:CcdB of 1:2. In the latter case, DNA eluted next to the CcdA:CcdB peak, and no CcdA or CcdB was found to bind on the DNA as evaluated with C4-RPC. This observation reflects the preference of CcdA to form a CcdA₂CcdB₂ complex over a DNA-CcdA-CcdB complex. We cannot explain the fact that we did not observe any DNA binding at this ratio, because based on simple equilibrium arguments one would expect at least a small fraction of CcdA-CcdB bound to DNA.

DNA peaks were evaluated on an ethidium bromide-stained 6% native DNA gel. A retarded migration was observed for the DNA–CcdA:CcdB 1:1 mixture compared with the free DNA, proving the binding of a CcdA–CcdB complex to the DNA and confirming the observed peak shift in the size-exclusion chromatography experiment. The ratio of CcdA:CcdB bound to this DNA fragment was determined with C4-RPC. Peak height calculation resulted in a clear 1:1 ratio of CcdA and CcdB bound to DNA (inset of Fig. 8). Hence, these experiments confirm the specific binding of a (CcdA₂CcdB₂ₙ) complex (n = 1, 2, 3, . . .) to the DNA operator/promoter region.

**Discussion—**Comparisons with other addiction systems are difficult because of the limited information that is available. Information regarding antidote stability and DNA binding is available for Phd/Doc on plasmid P1 (31, 34–36), parDE on plasmid RK2 (37, 38), and the maz system on the E. coli chromosome (39). The general properties of the regulatory aspect of these systems are very similar in all cases: the antidote is the main DNA-binding protein, and the presence of the toxin enhances its affinity to operator DNA. The details of these interactions on the other hand differ markedly among the different systems. In the parDE system, the antidote ParD alone is sufficient for repression and produces a clear DNase I footprint in the absence of the toxin ParD (38). The protected region in the ParD footprint (30–45 bp depending on the protein concentration used) is significantly smaller than the one we observe for the Ccd proteins. The maz system on the other hand requires both toxin and antidote to produce protection against DNase I, similar to ccd. The protected region in maz is only 47 bp (39) similar in length to parDE.

In all systems, one or more palindrome sequences are crucial for DNA binding, but the details differ again from system to system. ccd has a single 6-bp palindrome that seems to be the nucleation point for the binding of several CcdA₂ dimers along a 129-bp-long stretch. In the phd/doc system, two distinct palindromes are present, both of which bind Phd, but with different affinities (31). The binding site in the control region of the maz DNA on the other hand shows two overlapping palin-
dromes (termed “alternating palindromes” by the authors), both of which bind the Maz proteins (39).

**The CcdB-CcdA Interaction Is Unusually Complex**—Addiction systems are commonly found on stable low copy number plasmids (1). Only in the case of **ccd** and **kis/kid** (plasmid R1) the target of the toxin is known (14, 17). The **ccd** system is unique in the sense that a CcdB-resistant strain is available in which the toxin can be overexpressed in large amounts (14, 27). Hence it is possible to analyze the interaction between the addiction toxin and the antidote of this system in detail using biochemical and biophysical techniques. The interaction between CcdA and CcdB turned out to be surprisingly complex. Instead of forming a single complex that both acts as a repressor of the synthesis of the **ccd** proteins and protects gyrase from CcdB, a large variety of complexes seem to be possible. On the one extreme there is a soluble hexameric CcdA<sub>2</sub>CcdB<sub>4</sub> complex. The other extreme is a precipitate with a 1:1 molar ratio of the **ccd** proteins that probably consists of long chains of alternating CcdA and CcdB dimers.

Different experimental techniques require different concentration ranges. These concentrations are often not those that are relevant in vivo. Especially when studying complex phenomena such as the CcdA-CcdB interaction, where it is not possible to obtain accurate association constants, one should be careful when extrapolating experimental measurements to in vivo situations. The concentrations of CcdA and CcdB that are present in a bacterial cell are not known, but will certainly be much lower than those used in for example a calorimetric titration. Driving concentrations into unrealistic proportions and amounts, as compared with the in vivo situation, may unveil intrinsic properties of the molecular elements of the system. Moreover CcdA and CcdB do not form an isolated system in vivo. They have the possibility of interacting with each other as well as with other components in the system such as gyrase, gyrase-DNA complexes, specific operator DNA, and perhaps other promoter regions as well. Some of the phenomena observed in vitro may therefore be irrelevant in vivo.

**The Possibility of Generating Multiple Types of CcdA:CcdB Complexes May Fine-tune the Self-regulation of the ccd System**—The precipitate formed at 1:1 molar ratios of CcdA:CcdB is probably such a phenomenon arising from the high concentrations used in biophysical experiments as well as of taking the proteins outside their in vivo context. All complexes with a 1:1 ratio are fully bound to operator DNA. The difference between a 1:1 molar ratio when bound to DNA and a 1:2 ratio in solution may contribute to the finer specificity of the regulation of this system. With very low concentrations within the cell, even small fluctuations in the ratio of CcdA and CcdB, due to the actions of the Lon protease, may result in accidental poisoning of gyrase.

By allowing CcdB to extract a 1:1 CcdA:CcdB complex from its operator DNA, freed CcdB will remain inactive against gyrase and at the same time induce the synthesis of additional CcdA to restore the normal physiological balance (30). Note that this extraction is not in our reported Dnase I and gel retardation experiments (Figs. 5A and 6B), because we used very low amounts of DNA. Nevertheless in certain concentration regimes we also observed this extraction phenomenon corroborating the data, reported in detail by Afif et al. (30).

**Open or Closed Nature of the CcdA:CcdB Interaction**—The
posibility of having CcdA:CcdB complexes with different stoichiometries and the observation of two distinct affinity constants intrinsically raises a question concerning the three-dimensional architecture of these complexes. Since both CcdA and CcdB are homodimers, two fundamentally different types of complexes can be envisaged: open or closed (Fig. 9).

In an open complex, the interaction would only require a single type of interface and thus a single binding constant. This apparent contradiction with our experimental data is not fit with the inability of the soluble CcdA2CcdB4 to interact with promoter DNA.

In a closed complex, the molecular 2-fold axes of the CcdA and CcdB dimers interstrip. This is the most common way two dimeric proteins are expected to interact (Fig. 9). Since CcdA and CcdB have been shown to form long chains, this would mean that there are two distinct CcdA-CcdB interfaces (one on “the front” of CcdA interacting with “the back” of CcdB and vice versa). To explain the positive cooperativity in the system, the binding site on the “back” CcdA can only form as the consequence of a conformational change after a first CcdB dimer has bound to the front.” This unusual situation fully explains the existence of two binding constants, the potential of CcdB to act with promoter DNA.

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