The Action of the Bacterial Toxin Microcin B17

INSIGHT INTO THE CLEAVAGE-RELIGATION REACTION OF DNA GYRASE*

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We have examined the effects of the bacterial toxin microcin B17 (MccB17) on the reactions of Escherichia coli DNA gyrase. MccB17 slows down but does not completely inhibit the DNA supercoiling and relaxation reactions of gyrase. A kinetic analysis of the cleavage-religation equilibrium of gyrase was performed to determine the effect of the toxin on the forward (cleavage) and reverse (religation) reactions. A simple mechanism of two consecutive reversible reactions with a nicked DNA intermediate was used to simulate the kinetics of cleavage and religation. The action of MccB17 on the kinetics of cleavage and religation was compared with that of the quinolones ciprofloxacin and oxolinic acid. With relaxed DNA as substrate, only a small amount of gyrase cleavage complex is observed with MccB17 in the absence of ATP, whereas the presence of the nucleotide significantly enhances the effect of the toxin on both the cleavage and religation reactions. In contrast, ciprofloxacin, oxolinic acid, and Ca²⁺ show lesser dependence on ATP to stabilize the cleavage complex. MccB17 enhances the overall rate of DNA cleavage by increasing the forward rate constant (k₂) of the second equilibrium. In contrast, ciprofloxacin increases the amount of cleaved DNA by a combined effect on the forward and reverse rate constants of both equilibria. Based on these results and on the observations that MccB17 only slowly inhibits the supercoiling and relaxation reactions, we suggest a model of the interaction of MccB17 with gyrase.

Microcins are a family of toxins produced by Enterobacteriaceae to inhibit phylogenetically related species (1). They differ from colicins by their smaller molecular mass and production to inhibit phylogenetically related species (1). They differ

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The action of microcin on sensitive Escherichia coli cells leads to an arrest of DNA replication and, consequently, to the induction of the SOS response (7). Moreover, MccB17 induces, in vitro and in vivo, double-stranded cleavage of DNA mediated by DNA gyrase (8). A mutation in the B subunit of gyrase (W751R) was found to confer resistance to MccB17, confirming that DNA gyrase is a cellular target of the toxin (8). MccB17 blocks gyrase by trapping the enzyme-DNA cleavage complex, a mode of action reminiscent of that of quinolones (8, 9). Of the two pairs of tandem, fused 4,2-bisbicyclics in MccB17, the integrity of the second one (B-site tandem; Fig. 1A) was found to be crucial for the potency of MccB17 (10, 11).

DNA gyrase is a prokaryotic type II topoisomerase that negatively supercoils closed circular DNA with the requirement of ATP hydrolysis for energy (12, 13). The active enzyme is an A₂B₂ heterotetramer with the A subunit (GyrA, 97 kDa) largely responsible for DNA wrapping and the breakage and reunion of DNA and the B subunit (GyrB, 90 kDa) responsible for ATP hydrolysis and the interaction with GyrA and DNA. The enzyme introduces supercoils into DNA by wrapping a double-stranded segment around itself, cleaving this DNA (the gate segment) in both strands, passing the wrapped DNA (transported segment) through the break, and then resealing the DNA (12–15). Binding of ATP to GyrB causes the closure of the clamp formed by dimerization of GyrB, which captures the transported segment and directs it through the double-stranded break in the gate segment. ATP is then hydrolyzed, allowing the enzyme to return to its starting conformation (16–20).

In addition to MccB17, other inhibitors of DNA gyrase include the antibacterial agents coumarins and quinolones and the bacterial toxin CcdB (21–23). Coumarins competitively inhibit ATP hydrolysis, whereas quinolones, such as ciprofloxacin (CFX) and oxolinic acid (OXO), stabilize the covalent gyrase-DNA cleavage complex. CcdB also stabilizes a cleavage complex but only in the presence of ATP (21). The trapping of the gyrase-DNA cleavage complex by MccB17 is also reported to be ATP-dependent (9). DNA cleavage complexes can also be observed in the absence of drugs or toxins, if Mg²⁺ is substituted by Ca²⁺, with both gyrase (24) and eukaryotic top II (25).

The elucidation of the mode of action of MccB17 on DNA gyrase is important in at least two respects. First, because of its unusual chemical structure and properties, MccB17 may provide a model of a toxin-gyrase interaction, informative for new drug design. Second, key information on the mechanism of gyrase may be discovered from microcin action. In this paper, we study the effects of MccB17 on the kinetic parameters of the cleavage-religation reactions of E. coli DNA gyrase and compare the results with the effects on the supercoiling and relaxation reactions. By dissecting the cleavage and the religation...
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**FIG. 1. Effect of MccB17 on DNA supercoiling and relaxation by gyrase.**
A, chemical structure of MccB17. B, time course of supercoiling was followed at 25 °C with 2 mM ATP and 10 nM gyrase. C, time course of relaxation with 20 nM gyrase. The reactions were performed in the presence (+) or the absence (−) of 37.5 μM MccB17. R, relaxed DNA; SC, supercoiled DNA.

EXPERIMENTAL PROCEDURES

**Protein, DNA, and Drugs—**GyrA and GyrB were purified as previously described (26) and, with relaxed and supercoiled pBR322 DNA, were gifts from Mrs. A. J. Howells (John Innes Enterprises). DNA topoisomerase IV was a gift of Dr. M. K. Wall (John Innes Centre). Ciprofloxacin (Bayer) was dissolved in water. Microcin B17 and quinolones were gifts from Mrs. A. J. Howells (John Innes Enterprises). DNA topoisomerase IV was a gift of Dr. M. K. Wall (John Innes Centre). Ciprofloxacin (Bayer) was dissolved in water. Microcin B17 was produced from *E. coli* DH5α carrying the plasmid pUC19-mccB17 and purified and dissolved in Me2SO, according to the procedure previously described (10).

**Enzyme Assays—**Supercoiling assays were carried out as previously described (24), except that the reactions were incubated at 25 °C for various times up to 6 h and contained 21 nM relaxed pBR322, 10 nM gyrase, 2.5% (v/v) Me2SO, with or without 37.5 μM MccB17. Relaxation assays were performed as for supercoiling, with some modifications: ATP and spermidine were omitted, and the reactions included 7.1 nM negatively supercoiled pBR322 or 12.5 nM relaxed pBR322 was used as a substrate. The reactions included an excess of enzyme over DNA by a factor 4 or 10; 3.6 nM negatively supercoiled pBR322 or 12.5 nM relaxed pBR322 was used with 33–50 nM of enzyme. In the first method, following previous reports (25, 27–29), the Ca2+-induced cleavage reactions were brought to 300 mM NaCl to initiate the religation reactions, which were incubated on ice for various times up to 20 min. The second method was a modification of the Mg2+-promoted religation described elsewhere (25, 30). The cleavage reaction, on ice, was brought to 35 mM EGTA to chelate the Ca2+ ions, and the religation was induced by the presence of Mg2+ ions. If the religation assay was carried out with 2 mM ATP (or ADPNP), MgCl2 was added at the time of the cleavage reaction at a final concentration of 0.2 mM. When no nucleotide was present in the religation assay, MgCl2 was introduced at the same time as EGTA at a final concentration of 0.1 mM. The samples were collected for up to 40 min.

For each enzyme assay mentioned above, the DNA products were analyzed by electrophoresis through 1.0% agarose gels as previously described (9), and the fraction of DNA in the closed circular, nicked, and linear forms was quantified using the GeneTools software on a Syngene gel analysis system. All of the cleavage and religation assays were repeated several times, and typical data are shown in Figs. 2 and 3. Control experiments showed that the intensities of the DNA bands for the different forms of DNA were a true estimation of their concentration (data not shown).

**Kinetic Simulations—**Simulations modeling the observed kinetic curves were carried out using the KinTekSim software (KinTek Corporation) developed from the KINSIM and FITSIM programs (31, 32). Initial rate constant inputs were adjusted, using KINSIM and the mechanism shown in Scheme 1 (see below), until the simulated curves fitted the experimental data. Then the iterative nonlinear regression analysis FITSIM was used to predict how well the mechanism and set of rate constants describe the experimental data.

**RESULTS**

**DNA Supercoiling and Relaxation Reactions—**Previous work reported that MccB17 inhibits gyrase-catalyzed supercoiling slowly (11) or not at all (9) and that stabilization of the cleavage complex is ATP-dependent (9). To further investigate these effects, we have examined the gyrase supercoiling and relaxation reactions in the presence of MccB17 under a range of conditions.

In standard supercoiling reactions at 37 °C using 10–160 nM gyrase, the relaxed DNA substrate became completely negatively supercoiled, irrespective of the absence or the presence of
43 μM MccB17 (data not shown). The temperature of the reaction was reduced to 25 °C to decrease the rate of supercoiling and reveal the inhibitory effect of MccB17. With 15 nM gyrase, all relaxed DNA was supercoiled within 2 h without inhibitor versus 4 h if the reaction contained 37.5 μM MccB17 (data not shown). With 10 nM gyrase, complete supercoiling occurred within 6 h in the absence of inhibitor, whereas in the presence of MccB17, the reaction was slower and still incomplete after 6 h (Fig. 1B). An estimate of the inhibition of supercoiling by MccB17, from several experiments, suggests that MccB17 slows down the reaction by a factor of ~3. These data support the idea that MccB17 does not completely inhibit the gyrase supercoiling reaction.

The action of MccB17 on the relaxation reaction of gyrase was similar to that found with supercoiling. When relaxation of negatively supercoiled DNA was performed at 37 °C with 100 nM gyrase, relaxation was complete within 60 min, and no effect of MccB17 was observed (data not shown). Therefore, the reaction was slowed down by using 20 nM enzyme at 25 °C and observed over an extended period. Under these conditions, MccB17 slowly and partially inhibits the relaxation reaction of gyrase (Fig. 1C). The supercoiled DNA was quantified from Fig. 1C, as a function of time. After fitting data to a single exponential equation, the half-life of disappearance of supercoiled DNA was about three times greater in the presence of MccB17 than in its absence.

**DNA Cleavage**—The double-stranded breakage of DNA catalyzed by gyrase is normally transient (33) but can be trapped by quinolone drugs, Ca2+ ions, CcdB, or MccB17, resulting in a stable gyrase-DNA cleavage complex (9, 21, 24, 34, 35). The kinetics of Ca2+-, CFX-, OXO-, and MccB17-induced cleavage of relaxed DNA by gyrase were compared by taking samples at different times of reaction in the presence or the absence of ATP (Fig. 2A; OXO data not shown). The linear DNA species was quantified from the agarose gels; the amounts (%) were plotted as a function of time and fitted to a rectangular hyperbola; and the time to reach 50% of maximum cleavage was determined (Fig. 2, B and C). Fig. 2 represents a typical result from one experiment; the standard deviation from three different experiments with CFX, with and without ATP, was less than 15% (data not shown). The maximal amount of CFX-, OXO-, or Ca2+-induced cleavage at equilibrium was about 40% and was not greatly influenced by the presence of ATP in the reaction. The result was very different for MccB17, which induced a maximum of only 8% cleavage in the absence of ATP but up to 30% in the presence of the nucleotide. Similar to a previous study with CFX (9), the time taken to reach 50% of the maximal cleavage complex stabilized by CFX, OXO, or MccB17 was 3-fold longer in the absence of the nucleotide than in its presence. When Ca2+ was the cleavage-inducing agent, ATP did not influence the time to reach 50% of the maximal cleavage complex (Fig. 2).

In another approach, we varied the CFX or MccB17 concentration and determined the IC50 and maximum level of cleavage, with and without ATP, under the conditions described for Fig. 2 (data not shown). Consistent with previous results (9), we found approximately the same level of cleavage of relaxed DNA by gyrase, with and without ATP in the presence of CFX, but with IC50 values that differed by ~10-fold: 0.2 μM (with ATP) and 1.9 μM (without ATP). For MccB17 the maximum level of cleavage was ~10-fold greater in the presence of ATP than in its absence, and the approximate IC50 values were 1 μM (with ATP) and 17 μM (without ATP), confirming the strong dependence of MccB17 cleavage on the presence of ATP. As with relaxed circular DNA, the cleavage of linear DNA into smaller DNA fragments by gyrase in the presence of MccB17 was also greatly stimulated by ATP (9). Unexpectedly, we found that, in the absence of ATP, negatively supercoiled circular DNA was more efficiently cleaved by gyrase in the presence of MccB17 than relaxed circular DNA (data not shown). It seems that the ATP requirement for MccB17-induced cleavage depends on the topological state of the DNA.

**DNA Religation**—For religation assays, linear DNA was formed after 30 min of Ca2+-induced gyrase cleavage (30–40% of total DNA), and the amount of cleavage complex was not significantly influenced by the presence or the absence of nucleotide, quinolones or MccB17 in the reaction. In one method, the addition of a high concentration of salt (NaCl-induced religation) allowed cleaved covalent complexes to be religated while preventing the formation of new protein-DNA interactions. For topo II, the salt-induced reversibility of the calcium-induced cleavage is cation-dependent and prevented by prior addition of EDTA (25). Because the present salt-induced reaction was free of Mg2+, the religation occurred probably because of the presence of Ca2+ ions. In a second method (Mg2+-induced religation), chelation of the Ca2+ ions by excess of EGTA was performed, and the rate of religation depended on Mg2+ concentration and was too fast to be observed above 0.2 mM Mg2+ (data not shown). Time courses of religation were performed in the absence or presence of inhibitor (CFX, OXO, or MccB17), with and without ATP. Typical data for the Mg2+-induced religation are shown in Fig. 3.

The disappearance of linear DNA with time was quantified from panel A of Fig. 3 and plotted in panels B and C of Fig. 3. Time courses of linear DNA disappearance could be fitted to a
double exponential but not to a single exponential, indicating biphasic kinetics (Fig. 3, B and C). A similar experiment was carried out with the NaCl-induced religation (data not shown). CFX greatly inhibited the Mg$^{2+}$-induced religation reaction by 5- and 10-fold, respectively, regardless of the presence of ATP. CFX showed a similar ATP-independent inhibition in the NaCl-induced religation, except that it reduced primarily the second rate constant by 1 order of magnitude. In contrast with CFX, MccB17 required ATP for its inhibitory effect on religation; little or no inhibition is revealed in the absence of the nucleotide (Fig. 3). The MccB17 inhibitory effect was essentially due to a reduction in the second rate constant.

In the presence of ADPNP, MccB17 also showed an inhibition of the NaCl- and the Mg$^{2+}$-induced religations, although to a lesser extent than the reaction with ATP (data not shown). In contrast with CFX, OXO did not show any visible inhibition of the religation reaction in the absence of nucleotide and only a modest inhibition in the presence of ATP (Fig. 3C). In a control experiment, the amounts of cleavage induced by CFX and OXO were compared at various Mg$^{2+}$ concentrations; OXO was found to require twice as much Mg$^{2+}$ than CFX to stabilize 50% of maximum cleavage, and no OXO-induced cleavage was observed at the concentrations of Mg$^{2+}$ used in the religation assay (data not shown). Therefore, the absence of any effect of OXO on religation reactions can be explained by low binding of the drug to the gyrase-DNA complex at Mg$^{2+}$ concentrations below 0.5 mM.

Kinetic Simulations—The aim of the present work was to explain the effect of MccB17 on the cleavage-religation equilibrium of gyrase and to compare this effect with that of CFX, OXO, and Ca$^{2+}$. To explain the origin of the biphasic kinetics observed in the religation reactions, we propose a mechanistic scheme for the cleavage-religation reactions of gyrase. Earlier reports on gyrase and topo II suggested that both the cleavage and religation reactions occurred via a two-step process involving a nicked DNA intermediate (29, 30, 36). The first analysis of the kinetic curves of the cleavage and the religation reactions used a simple model of two irreversible consecutive reactions (29, 36). However, as mentioned above, the religation reaction does not fit a single exponential, suggesting the requirement for a more advanced scheme. In agreement with the early work on the eukaryotic topo II (30) and based on the fact that the cleavage-religation of DNA gyrase is an equilibrium process that can be reversed by the addition of salt (29) or the replacement of Ca$^{2+}$ by Mg$^{2+}$, we propose a kinetic scheme for the cleavage-religation reactions of DNA gyrase and other type II DNA topoisomerases that involves two-sequential equilibria.

Scheme 1

This scheme assumes that each molecule of DNA is bound to gyrase, because our assays contained an excess of enzyme over DNA. CC represents the closed circular DNA, OC represents the open-circular (single-stranded break) DNA, and L represents the linear (double-stranded break) DNA. $K_1$ ($K_{1}'$) and $K_2$ ($K_{2}'$) are the two equilibrium constants of the two-step cleavage (religation) reaction. Rate and equilibrium constants in religiation are given a primed designation to indicate that the cleavage and religation reactions are measured under different conditions, in particular at different Mg$^{2+}$ concentrations, and...
that the rate and equilibrium constants are not expected to be identical. Simulations using this scheme show that, by adjusting the values of the individual rate constants, the biphasic kinetics observed in Fig. 3 can be reproduced. Experimental data and their simulation for the cleavage and the religation reactions are displayed in Figs. 4 and 5, and the deduced kinetic constants are shown in Tables I and II, respectively.

The simulated curves for the cleavage induced by CFX, OXO, or Ca\(^{2+}\) were similar when ATP was present and only slightly different when ATP was absent (Fig. 4). In the reactions with these three compounds, the equilibrium constants, \(K_1\) and \(K_2\), were little influenced by the presence of the nucleotide (Table I), and the macroscopic equilibrium constants (product of \(K_1\) and \(K_2\)) between the closed circular and the linear forms were comparable. The DNA cleavage reaction induced by MccB17 gave a very different picture; only a minimal amount of linear DNA was produced in the absence of ATP, as compared with that in the presence of the nucleotide (Fig. 4). This is reflected in the very different values of \(K_1\) and \(K_2\) in the absence and presence of ATP (Table I). The effect of MccB17 on the cleavage reaction with ATP was due to an increase of \(K_2\) by 1 order of magnitude (because of corresponding increase in \(k_2\)), whereas the first equilibrium constant, \(K_1\), was unaffected.

Only the simulations of the religation induced by Mg\(^{2+}\) is presented here (Fig. 5), but results with the NaCl-induced reaction are consistent with the following findings. The religation assay is a more complex mixture than the cleavage assay, but the kinetic curves were easier to compare because the religation could be performed both in the absence and in the presence of inhibitors. MccB17 was able to efficiently inhibit religation only when ATP was present (Fig. 5). Remarkably, as in the cleavage assay, the effect of MccB17 was largely caused by an increase of \(k_2\) and hence \(K_2\), indicating that the toxin

Fig. 5. Kinetic simulation of religation reactions. Religation assays contained either no inhibitor, 37.5 \(\mu\)M MccB17, or 25 \(\mu\)M CFX. Intensity of the linear (●), open circular (○), and closed circular (▼) bands was quantified from Fig. 3A and plotted as a percentage of total DNA. The closed and open circular forms were corrected for the nicked DNA population in the DNA alone. The reactions were performed without (left panels) or with (right panels) 2 mM ATP. The simulated curves were obtained using KinTekSim and Scheme 1, and the derived rate constants are given in Table II.

| ATP | \(k_1\) | \(k_{-1}\) | \(k_2\) | \(k_{-2}\) | \(K_1\) | \(K_2\) | \(K_1K_2\) |
|-----|--------|--------|--------|--------|-------|-------|----------|
| No inhibitor | 0.25 | 1.27 | 0.02 | 0.6 | 0.2 | 0.033 | 0.007 |
| + MccB17 | 0.11 | 0.56 | 0.2 | 0.8 | 0.2 | 0.25 | 0.05 |
| + CFX | 0.08 | 0.8 | 4.4 | 1.8 | 0.1 | 2.4 | 0.24 |
| + OXO | 0.08 | 0.32 | 0.13 | 0.2 | 0.25 | 0.65 | 0.16 |
| + MccB17 + OXO | 0.35 | 1.25 | 0.14 | 0.15 | 0.28 | 0.93 | 0.26 |

* 3% (v/v) Me\(_2\)SO was present in the cleavage assay.
+ 2.5 mM KOH was present in the cleavage assay.

Table II

Effects of MccB17, CFX, and OXO on the kinetic parameters of the Mg\(^{2+}\)-induced religation reaction of DNA gyrase.

| ATP | \(k_1\) | \(k_{-1}\) | \(k_2\) | \(K_1\) | \(K_2\) | \(K_1K_2\) |
|-----|--------|--------|--------|-------|-------|----------|
| Ca\(^{2+}\) | − 6.9 | 48.5 | 2.2 | 0.6 | 0.14 | 3.7 | 0.52 |
| + CFX | 4.4 | 29.4 | 3.6 | 0.8 | 0.15 | 4.5 | 0.68 |
| + MccB17 | 2.8 | 12.4 | 1.0 | 0.3 | 0.23 | 3.3 | 0.76 |
| — OXO | 4.4 | 28.6 | 3.3 | 0.7 | 0.15 | 4.7 | 0.71 |

FIG. 4. Kinetic simulation of cleavage reactions. Cleavage assays contained either 4 mM Ca\(^{2+}\), 25 \(\mu\)M CFX, 37.5 \(\mu\)M MccB17, or 500 \(\mu\)M OXO. Intensity of the closed circular (●), open circular (○), and linear (▼) bands was quantified from Fig. 2A and plotted as a percentage of total DNA. The closed and open circular forms were corrected for the nicked DNA population in the DNA alone. The reactions were performed without (left panels) or with (right panels) 2 mM ATP. The simulated curves were obtained using KinTekSim and Scheme 1, and the derived rate constants are given in Table I.
affects the cleavage-religation equilibrium primarily by increasing the forward rate of cleavage (Table II). In contrast with MccB17, CFX increased mainly, but not exclusively, $K_2$ by increasing $k_+$ and decreasing $k_{-2}$, indicating that CFX both increased the forward rate of cleavage and decreased the reverse rate of religation (Table II). To investigate the action of the toxin on $k_2$, several time courses of Mg$^{2+}$-induced religation were performed at various concentrations of MccB17, whereas Me$_2$SO was kept constant at 2% (v/v). Simulations using the same kinetic scheme confirmed that only $k_2$ and $K_2$ increased with increasing amount of the toxin (Fig. 6); $k_-$ and $K_1$ were largely independent of MccB17 concentration.

**DISCUSSION**

*Effect of MccB17 on Supercoiling and Relaxation*—Previous work on the effect of MccB17 on DNA supercoiling catalyzed by DNA gyrase suggested that MccB17 is an inefficient inhibitor of the enzyme (9, 11). In this work we found that the inhibition by 37.5 μM MccB17 is slow and incomplete, i.e. we have not found conditions where the reaction is completely prevented, even at a high concentration of the toxin (Fig. 1). We investigated the effect of MccB17 on ATP-independent relaxation by gyrase and found that the inhibition of this reaction by the toxin was also slow and incomplete. These results show that MccB17 inhibits supercoiling and relaxation of gyrase to a similar extent, and contrary to earlier reports (9, 11), the action of MccB17 is not ATP-dependent. In contrast with MccB17, quinolones were shown to rapidly and completely inhibit the supercoiling and relaxation reactions of gyrase (36–38). We suggest below that the mode of action of MccB17 on gyrase is distinct from that of quinolones and may involve inhibition of the strand passage process. We found also that MccB17 can inhibit the ATP-dependent relaxation reaction of topo IV (data not shown), suggesting that MccB17 is not absolutely specific to gyrase.

*Effect of Gyrase Inhibitors on the Cleavage-Religation Equilibrium*—In previous work, MccB17 was found to show little or no stabilization of the gyrase-DNA cleavage complex in the absence of ATP (9). In the present work, we confirmed that, without ATP and using relaxed DNA as substrate, a low amount of cleavage product is formed (Fig. 2). However, in the presence of ATP, the cleavage product is increased to a level comparable with that obtained with other agents. Interestingly, with negatively supercoiled DNA as substrate, substantial DNA cleavage occurred without ATP. When we examined religation, we found that MccB17 showed only low level inhibition without ATP but that in the presence of the nucleotide it was comparable in potency to CFX (Fig. 3). The observed religation kinetics were biphasic, suggesting that the process cannot be described as two consecutive irreversible reactions, as previously reported (29). However, when we treated the cleavage-religation process as two consecutive equilibria (Scheme 1), both the cleavage and religation data could be accommodated. In this scheme, the cleavage and religation reactions are not treated as mutually exclusive but as concomitant, i.e. in true equilibrium, similar to a scheme proposed for *Drosophila* topo II (30). In fact, inspection of data from earlier work (29, 39) reveals that religation was not readily fitted using single exponentials and that our Scheme 1 would be a more suitable model.

The rate and equilibrium constants derived from this scheme allow us to deduce the effect of different agents on the individual steps of the cleavage-religation process. However, caution must be exercised in the interpretation. We are globally determining four rate constants from fitting three relative concentrations (CC, OC, and L species; Figs. 4 and 5), and experimental errors (gel-based assay) may have disproportionate impacts on the value of some rate constants. The small systematic drift observed in the data in Fig. 5 suggests a slow conversion of nicked circular to closed circular DNA. We were unable to discern the origin of this phenomenon, but several identical experiments showed that this drift did not significantly change the results from Table II. Moreover, when the measured concentrations of the species are low (e.g. OC and L species in cleavage with MccB17 in the absence of ATP; Fig. 4), the derived rate constants are likely to be less reliable. Also it should be borne in mind that the religation assays (Fig. 3) have to be performed under different conditions from the cleavage assays (e.g. Mg$^{2+}$ concentration) and that the derived kinetic
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parameters from the two assays cannot be compared directly. Nonetheless, from this analysis, it is possible to infer some general conclusions about the effects of MccB17 and other inhibitors on gyrase.

From Tables I and II, it can be seen that, with relaxed DNA as substrate, MccB17 does not efficiently stabilize the gyrase-DNA cleavage complex in the absence of ATP, as judged by the values of \( k_1 \) and \( k_2 \). In the presence of ATP, the cleavage complex is significantly stabilized by MccB17, which is attributable principally to an increase in \( k_c \) (Fig. 6). Even in the absence of ATP, where the level of cleavage is low with relaxed DNA, the comparison of the recombination kinetics with and without toxin suggests that the same step, \( k_2 \), is affected.

The quinolone drugs, CFX and OXO, are distinctly different in their effects compared with MccB17 and also show differences from each other. Overall, both drugs stabilized the cleavage complex in cleavage reactions (Table I), regardless of the presence or the absence of ATP. Using the MccB17 cleavage reaction in the absence of ATP as a pseudo-reference, CFX affects both \( k_1 \) and \( k_2 \), whereas OXO principally affects \( k_2 \). As regards recombination, CFX has an effect comparable with that in the cleavage reaction, although in this case largely \( k_2 \) is affected.

The action of CFX on the gyrase cleavage-religation equilibrium resembles that observed on topo IV, where the rate of DNA cleavage is increased and the religation of cleaved DNA inhibited (40), but differs from the eukaryotic topo II, where quinolones enhance the forward rate of cleavage with little effect on religation. CFX-induced cleavage could be attributable to the reaction conditions, e.g., the low Mg\(^{2+}\) concentration, leading presumably to weaker binding of OXO to gyrase.

Taken together it appears that CFX action is largely ATP-independent and mostly, but not exclusively, affects the second step of the equilibrium (\( k_1 \) or \( k_2 \)). However, it should be noted that under other reaction conditions, significant effects of ATP on CFX-induced cleavage can be observed (9, 43), but it is clear that these effects are very dependent on conditions. In terms of individual rate constants, there is not a particular step that predominantly changes in the presence of CFX, unlike the situation with MccB17. Ca\(^{2+}\) was only tested in the cleavage reaction; like CFX, its effect is largely ATP-independent, and it appears to affect both equilibria, \( k_1 \) and \( k_2 \), to a similar extent. It is expected that the effect of Ca\(^{2+}\) is due to its substitution for Mg\(^{2+}\) in the cleavage-religation process.

Interaction of MccB17 with Gyrase—From our data and comments on the manuscript, it seems likely that MccB17 interacts with the gyrase-DNA complex in a manner distinct from quinolones. Further experiments will be required to determine the molecular details of this interaction.

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