Preferential Binding of the Archaebacterial Histone-Like MC1 Protein to Negatively Supercoiled DNA Minicircles

Caroline Teyssier,‡ Francine Toulmé,‡ Jean-Pierre Touzel,§ Alain Gervais,‡ Jean-Claude Maurizot,‡ and Françoise Culard*,‡

Centre de Biophysique Moléculaire, rue Charles Sadron, 45071 Orléans Cedex 2, France, and INRA, LGPTA, BP39, 59651 Villeneuve d'Ascq Cedex, France

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ABSTRACT: The interaction of the archaebacterial MC1 protein with 207 bp negatively supercoiled DNA minicircles has been examined by gel retardation assays and compared to that observed with the relaxed DNA minicircle. MC1 binding induces a drastic DNA conformational change of each minicircle, leading to an increase of the electrophoretic mobility of the DNA. A slight increase in salt concentration enhances the amount of bound MC1, and high NaCl concentrations are required to dissociate the complexes. Furthermore, the salt effect on binding depends on the supercoiling state of the DNA. The dissociation rates decrease with increasing linking difference of the minicircles relative to their relaxed configuration to reach a maximum at −2 turns. In addition, differences between the topoisomers are also observed in terms of stoichiometry of the strongest complexes. So with the −2 topoisomer the complex with two MC1 molecules is the most stable, while with the −1 and −3 topoisomers, the strongest ones are those with one MC1 molecule per DNA ring.

Numerous proteins have been found associated with prokaryotic nucleoids. These abundant, small, and usually basic proteins are generally referred to as histone-like proteins as they are considered to play roles analogous to those of eukaryotic histones (Drlica & Rouvière-Yaniv, 1987).

MC1 is the most abundant histone-like protein present in various species of *Methanosarcinaceae* (Chartier et al., 1985). In the *Methanosarcina* sp. strain CHTI 55, MC1 is a 93 amino acid polypeptide, exhibiting a marked hydrophilic character provided by a large number of basic and acid residues (respectively 24 and 12) and no hydrophobic domain (Chartier et al., 1989). MC1 preferentially binds to double-stranded DNA as a monomer in a noncooperative way (Culard et al.,1993). It can protect DNA against thermal denaturation (Chartier et al.,1988) and against radiolysis by fast neutrons (Isabelle et al., 1993).

Electron microscopy experiments have shown that MC1 binding does not induce a DNA wrapping around the protein like the core of histones (Toulmé et al., 1995). On the other hand, significant changes in DNA structure are observed upon MC1 binding. These changes include formation of DNase I hypersensitive sites, sharp DNA bending, and compaction of relaxed circular DNA (Laine et al., 1991; Teyssier et al., 1994; Toulmé et al., 1995).The significance of these changes in the DNA structure seems to suggest that, as a corollary, DNA supercoiling may alter MC1 binding.

In this paper we have used DNA minicircles that are a powerful tool to study the topology of DNA both free and complexed to proteins (Zivanovic et al., 1988; Lobell & Schleif, 1990; Sasmor & Betz, 1990; Gruskin & Rich, 1993; Kahn & Crothers, 1992; Lavigne et al., 1994). Three 207 bp[1] supercoiled minicircles, differing from a relaxed DNA minicircle by a reduction of one, two, and three in their linking number, were prepared. Comparison of MC1 binding to these topoisomers by gel retardation experiments and quantification of the stability of the various complexes underline the importance of the topological state of the DNA in MC1 binding.

MATERIALS AND METHODS

DNAs. The 207 bp DNA fragment containing the *lac* promotor−operator region was isolated from a plasmid derived from pBR 322 by *Eco*RI restriction cleavage (Fuller, 1982). It was 5′ end-labeled by T4 polynucleotide kinase with [γ^{32}P]ATP, followed by a chase with cold ATP. DNA concentrations were determined by absorption spectrophotometry using an extinction coefficient of 13 000 M^{-1} cm^{-1} per bp at 260 nm.

MC1 Protein. *Methanosarcina sp.* CHTI 55 (DSM 2906) was grown as indicated in Chartier et al. (1985). Protein MC1 was prepared as previously described (Laine et al., 1991). Its concentration was determined by absorption spectrophotometry using an extinction coefficient of 11 000 M^{-1} cm^{-1} at 280 nm.

Circularization and Topoisomer Identification. To obtain DNA minicircles, 207 bp labeled DNA fragments were incubated at a concentration of 0.11 μg/mL in ligase buffer (66 mM Tris HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTE) in the presence of 20 units of T4 DNA ligase/mL for 120 min at 25 °C. For the negatively supercoiled topoisomers, ligations were performed in the presence of ethidium bromide at 0.4, 0.8, and 1.6 μg/mL. After phenol−chloroform extraction and ethanol precipitation, samples were loaded, at room temperature, on preparative polyacrylamide

* To whom correspondence should be addressed. Tel: +33 38 51 55 64. Fax: +33 38 63 15 17. E-mail: culard@cnrs-orleans.fr.
‡ Centre de Biophysique Moléculaire.
§ INRA, LGPTA.
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[1] Abbreviations: bp, base pair(s); 3 D, three dimensional.

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gels (4% acrylamide, 0.2% bisacrylamide) in the presence of 0.6 μg of ethidium bromide/mL in the gel and the buffer (TBE = 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8). DNA minicircle molecules were extracted from gel slices, purified by Prepac chromatography columns (BRL) and phenol extractions, and finally dialyzed against TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The presence of the intercalating agent during the electrophoresis allows the separation of nicked (the most retarded) and closed DNA minicircles that migrate according to their linking number (Horowitz & Wang, 1984).

The calculated twist of an unconstrained DNA minicircle (Tw_0) can be obtained through the relation $Tw_0 = N/h_0$ where N is the ring size, 207 bp in our case, and h_0 is the number of bp per helical turn. Taking a value of 10.5 bp per turn for h_0 (Horowitz & Wang, 1984; Goulet et al.,1987) leads to a Tw_0 of 19.7. As the linking number (Lk) must be an integer and according to Lk = Tw + Wr (with Wr, the writhe, equal to 0), we estimate that for the single DNA minicircle obtained in absence of torsional constraint (called relaxed DNA) Lk is 20. Therefore, this DNA minicircle, also called 0 topoisomer, is probably slightly positively supercoiled. The negatively supercoiled topoisomers, called −1, −2, −3 topoisomers, have linking numbers of 19, 18, and 17, respectively. Their superhelical densities (σ), calculated according to $\sigma = (Lk - Tw_0)/Tw_0$ (Bates & Maxwell, 1989) were −0.036, −0.086, and −0.137, respectively.

Gel Retardation Assays. Complexes were prepared by mixing DNA minicircles (100 pM) and MC1 protein (as indicated) in TE buffer to a final volume of 15 μL. The mixture was equilibrated for 15 min at room temperature prior to loading on the gel. To study the salt effect, MC1 and DNA minicircles were incubated in a 105 μL volume in TE buffer. After equilibration, the mixture was divided in portions of 15 μL. Then 2 μL of a solution containing TE buffer or TE buffer plus NaCl at different concentrations was added, and the mixture was again incubated for 15 min. In both cases samples were gently mixed with 1/5 (v/v) of loading buffer (0.01% bromophenol blue, 0.01% xylene cyanol, 50% glycerol in TE) and loaded on a polyacrylamide gel (4% acrylamide/0.2% bisacrylamide) in TBE buffer. Prerun and run of the electrophoresis were performed at 10 V per cm. The gels were dried and autoradiographed with CGR films. The relative amount of DNA in each band was quantified using a β scanner (PhosphorImager, Molecular Dynamics).

Measurements of Dissociation Rates. Dissociation rate constants were essentially determined as in Toulmé et al. (1995). Briefly, DNA minicircles (100 pM) and protein MC1 (at various concentrations) were mixed in different salt conditions as indicated. After equilibration for 15 min, a 50 000-fold excess of unlabeled supercoiled pUC8 plasmid DNA was added as a competitor. After the indicated times, dissociation was stopped by loading a portion of the incubation mixture onto a running gel. As previously observed (Toulmé et al., 1995), the absence of further dissociation on the gel was ensured by the fact that we did not observe any smear or loss of DNA. Furthermore the bands remained thin. The percentage of bound DNA was determined for the various time points, and the half-lives were obtained as described by Riggs et al. (1970).

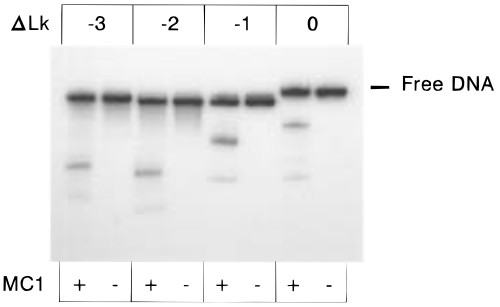

FIGURE 1: Electrophoretic mobilities of the DNA minicircles in the absence (−) and in the presence (+) of MC1 at a protein to DNA ratio of 2. The linking number difference (ΔLk) of DNA minicircles is indicated above the lanes. The samples (in TE buffer) were loaded on a polyacrylamide gel (4% acrylamide, 0.2% bisacrylamide in TBE buffer). For each topoisomer, the different DNA bands are, from top to bottom, free DNA, complex C1, and complex C2.

RESULTS

MC1 Compacts the Negatively Supercoiled DNA Minicircles. Figure 1 shows the electrophoretic mobilities of the DNA minicircles in the absence and in the presence of MC1. The free supercoiled topoisomers (ΔLk = −1, −2, and −3) co-migrate through the gel, and the free relaxed minicircle (ΔLk = 0) is retarded to a low extent. On the other hand, the MC1-bound minicircles are quite well separated. In the binding conditions used, low MC1−DNA ratio ($R = 2$) and low ionic strength (TE buffer), two different complexes are formed with each topoisomer. Both migrate faster than the free DNA. In each case, as previously shown with the relaxed minicircle (Toulmé et al.,1995), the C1 complexes (one MC1 molecule per minicircle) and the C2 complexes (two MC1 molecules per minicircle) are accelerated. The highest mobilities are observed for the C2 complexes with the −2 topoisomer.

At higher protein-DNA ratios, complexes with three (C3), four (C4), and more proteins are also formed. From C2, the mobility of the complexes is that classically observed by retardation gel for a nonspecific binding. A ladder of retarded bands is obtained, so that C2 migrates faster than C3, which migrates faster than C4, and so on (not shown).

Different conclusions can be drawn. (i) under these conditions, the free supercoiled minicircles, which are not separated on the gel, have similar shapes with little or no writhe. (ii) As previously discussed for the relaxed DNA (Toulmé et al., 1995), the MC1 binding should only lead to DNA retardation by increasing the frictional coefficient and by partial charge neutralization. The enhancement of the DNA mobility through the gel must therefore be due to a DNA conformational change inducing a reduction of the hydrodynamic volume of each DNA minicircle. Two levels of DNA compaction, induced by the binding of one and two MC1 molecules, are observed regardless of the supercoiling of the DNA minicircles. The most compact-shaped DNA minicircle is obtained for the complex C2 with the −2 topoisomer.

Effect of Salt on Binding. We have previously shown that a slight salt increase enhances the MC1 binding to the relaxed DNA minicircles (Toulmé et al., 1995). We have now studied the effect of NaCl addition on the MC1-topoisomeric minicircles complexes. As these experiments have been done within a large range of NaCl concentration (up to 500 mM),

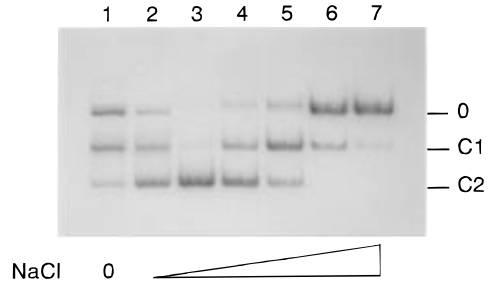

FIGURE 2: Effect of salt addition on the complexes of MC1 with the −1 topoisomer. MC1 (400 pM) and −1 topoisomer (100 pM) were mixed in TE buffer. The mixture was equilibrated and divided in aliquots, and TE or TE plus NaCl was added. Final NaCl concentrations were as follows from lanes 1 to 7: 0, 60, 120, 175, 235, 355, and 470 mM. The various mixtures were separated on a retardation gel (same composition as in Figure 1). Positions of free DNA (0), complex C1, and complex C2 are shown.

the dissociation of the complexes has also been studied. With each individual topoisomer, a pool including free DNA, complex C1 (about 30% of the DNA), and C2 (about 15% of the DNA) was formed and divided in aliquots. Then a small quantity of different NaCl solutions was added, and the various mixtures were loaded on retardation gels.

As an example of such an experiment, Figure 2 shows a retardation gel with the −1 topoisomer. The amount of complex C2 increases up to about 120 mM NaCl (lane 3), and then it decreases at higher NaCl concentrations. First, one MC1 molecule is dissociated from complex C2 to form complex C1 (lanes 4−5), and then, at even higher NaCl concentration, the complex C1 is dissociated, releasing the free DNA (lanes 6−7).

A quantitative analysis of the salt effect with each of the four DNA minicircles is shown in Figure 3. The relative amounts of each complex (expressed in DNA %) were plotted against NaCl concentration. MC1 shows a higher affinity for the supercoiled than for the relaxed minicircles. Indeed, with the relaxed topoisomer all DNA molecules are free at 200 mM NaCl whereas higher salt concentrations are required to dissociate the complexes with the supercoiled DNA minicircles. With all topoisomers, two parts can be distinguished. First, a slight salt addition enhances the amount of bound MC1. Second, at higher NaCl concentrations the dissociation of the complexes is observed. However, each topoisomer has a particular behavior, and the stoichiometries of the complexes differ.

With the relaxed topoisomer, the amount of complex C2 is enhanced up to 100 mM NaCl. The two MC1 molecules then dissociate simultaneously, and very little complex C1 is formed.

With the −1 topoisomer, as with the relaxed minicircle, the amount of complex C2 increases with the salt concentration but the dissociation steps differ. Indeed, decrease of complex C2 is accompanied by an increase of C1 up to 250 mM NaCl.

With the −2 and −3 topoisomers a slight NaCl addition enhances the amount of C2 complexes, but with those two topoisomers there is also formation of C3 complexes (three MC1 molecules per DNA minicircle). The C3 complexes begin to dissociate at about 120 mM NaCl. Then the pattern of dissociation with those two topoisomers differs. With the −3 topoisomer, the complex C2 begins to dissociate by relatively low NaCl concentrations (150 mM), leading to an increase in the complex C1, while with the −2 topoisomer

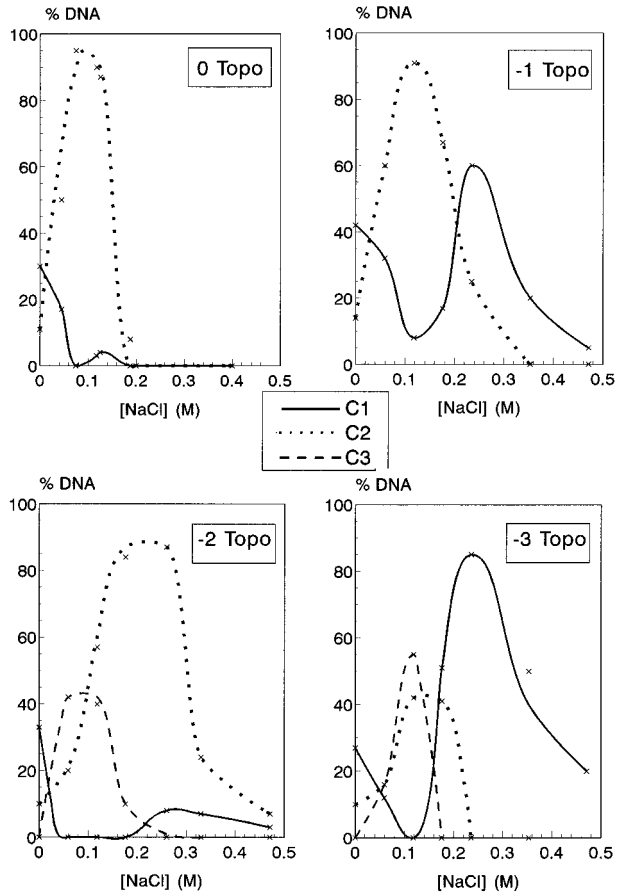

FIGURE 3: Quantitative analysis of salt effect on the MC1−topoisomer complexes. For each individual topoisomer, the percentage of DNA in each species was determined from retardation gels (as in Figure 2). From these values we plotted the percentage of DNA in each complex versus NaCl concentration (M). The various complexes are, respectively, complex C1 (—), complex C2 (•••) and complex C3 (- - -). To clarify the figure, the percentage of free DNA is not shown.

the complex C2 is very strong. NaCl concentrations up to 250 mM are required to dissociate the two remaining MC1 molecules. With this topoisomer very little complex C1 is formed.

Stabilities of Various Complexes. In order to analyze the stability of the various complexes, we studied the dissociation rate of preformed complexes, at moderate ionic strength (100 mM NaCl), in the presence of a large excess of unlabeled competitor DNA. Different protein/DNA ratios were used in order to study all the different complexes. Dissociation was stopped by loading aliquots of the dissociation mixture on running gels. For the most stable complexes, several gels were necessary and the dissociation was followed up to 30 h. The amount of complexes was calculated as a function of the incubation time. The results for the various C2 complexes are shown in Figure 4. Identical dissociation rates were obtained with different levels of excess of competing DNA indicating that pseudo first order kinetics occurs. Clearly, the MC1 binding to DNA minicircles highly depends on DNA topology since the dissociation rates vary with the supercoiling of the DNA minicircles. Indeed, after 5 h most of the relaxed DNA molecules are free while about 70% of the DNA is still complexed in the case of the −1 topoisomer. The complexes are even stronger with the −2 topoisomer since the dissociation is barely visible. On the other hand, with the −3 topoisomer the complexes are less stable.

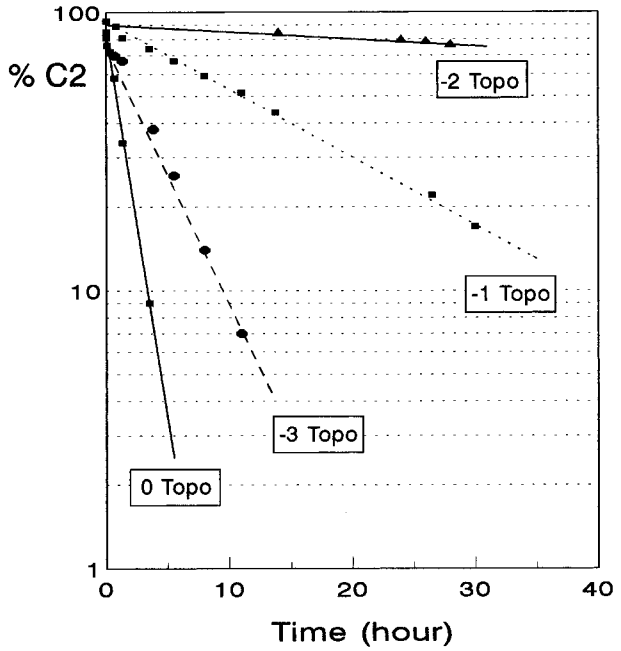

FIGURE 4: Rates of dissociation of preformed C2 complexes with 0, −1, −2, and −3 topoisomers. The DNA was first incubated in the presence of MC1 for 15 min (time 0). The incubation was continued in the presence of a 50 000 molar excess of supercoiled plasmid DNA. Aliquots of the mixture were loaded on gels at different times. From scanning of gels, the percentage of DNA in C2 complexes was determined and plotted with a logarithmic scale versus the incubation time for each topoisomer: $\Delta Lk = 0$ (−■−), $\Delta Lk = -1$ (·■·), $\Delta Lk = -2$ (−▲−); $\Delta Lk = -3$ (--●--). Binding buffer was 100 mM NaCl in TE. Each point results from three independent experiments.

Table 1: Half-Lives (in min) of Complexes of MC1 with DNA Minicircles Calculated from Logarithmic Plots as in Figure 4[a]

	ΔLk			
	0	−1	−2	−3
C1	*	>900	*	>900
C2	60	800	>900	280
C3	<1	<1	115	20

[a] Binding buffer was 100 mM NaCl in TE. *, complexes barely or not formed.

Table 1 shows the half-lives of various complexes calculated from dissociation curves at 100 mM NaCl. The half-lives of some complexes have not been obtained since they are not abundant whatever the MC1-DNA ratio. That is the case for the C1 complexes with the 0 and −2 topoisomers. For other complexes, dissociation is observed from the first time tested (1 min), therefore their half-lives are considered to be shorter than 1 min (C3 complexes with the 0 and −1 topoisomers). Finally, other complexes are too stable to be studied at this ionic strength. That is the case for the C1 complexes with the −1 and −3 topoisomers and for the complex C2 with the −2 topoisomer. To compare the stabilities of these very stable complexes, the same experiments were done at higher ionic strength (200 mM NaCl, 10 mM $MgCl_2$). As expected the complexes were less stable in those salt conditions. The maximal half-life ($\Gamma_{1/2}$) is observed for the complex C2 with the −2 topoisomer ($\Gamma_{1/2} = 280$ min) and then, in decreasing order, for the C1 complexes with the −1 topoisomer ($\Gamma_{1/2} = 60$ min) and with the −3 topoisomer ($\Gamma_{1/2} = 30$ min).

The two approaches used, the effect of salt on the complexes and the dissociation rate measurements, give consistent results. Indeed, NaCl addition favors the C3 complexes only with the −2 and −3 topoisomers, and these complexes are also relatively stable in 100 mM NaCl. Furthermore, the various complexes still present in high ionic strength (above 300 mM NaCl) are also those with the longest half-life (C1 with the −1 and −3 topoisomers, C2 with the −2 topoisomer).

DISCUSSION

MC1 is a small and abundant protein that bends DNA upon binding (Laine et al., 1991; Teyssier et al., 1994; Toulmé et al., 1995). These properties are shared with eubacterial histone-like proteins that presumably function by organizing an ordered three-dimensional nucleoprotein structure either alone or together with other proteins [for reviews see Schmid (1990) and Oberto et al. (1994)]. DNA minicircles can be considered as models for small constraint DNA domains caused by looping or synapsis in which histone-like proteins act as architectural elements (Segall et al., 1994; Paull & Johnson, 1995). It is therefore of interest to investigate how changes in the DNA topology of minicircles DNA affect MC1 binding.

As shown Figure 1 and Table 1, the mobilities and the stabilities of the complexes as well as the stoichiometries of the strongest complexes are altered by the supercoiling of the DNA minicircles. Previous studies of chromatin reconstitution on DNA minicircles have shown that a single change in linking number between two topoisomers has a great influence on the relative nucleosome uptake and on the stoichiometry of the reconstituted products (Goulet et al., 1988; Zivanovic et al., 1988). Surprisingly, MC1-minicircle interaction has similar properties, whereas MC1 is much smaller than the histone octamer and does not wrap DNA like histones (Toulmé et al., 1995).

In negatively supercoiled molecules, the linking number deficit (ΔLk) is partitioned into changes of DNA twist, Tw (duplex distortion) and writhe Wr (axis distortion). The presence of counterions which weaken repulsions between opposite segments cause an increase in the writhing component of the linking number deficit. In this way, Bednar et al. (1994) have shown that a 178 bp DNA minicircle with $\Delta Lk = -2$ undergoes a transition from a planar circle in low salt buffer to a writhed "figure-eight" conformation in the presence of high NaCl concentration. The minicircles are then composed of two diametrically opposed end loops characterized by strong helical curvatures. Therefore, it is likely that in the very stable C2 complexes with the −2 topoisomer the two MC1 molecules are located on these two loops. Interestingly, we have previously observed, by electron microscopy, that the two MC1 molecules are also diametrically opposed on the relaxed DNA minicircle (Toulmé et al., 1995). However, with the −1 and −3 topoisomers, the strongest complexes include only one MC1 molecule. In these cases, the DNA conformation at the opposite of the first MC1 molecule bound is probably not favorable for the binding of a second protein. With the −3 topoisomer, due to its relatively large superhelical density ($\sigma = -0.137$), some altered DNA structure is possible. It is clear that only accurate knowledge of the 3 D shape of these DNA minicircles would lead to a definitive interpreta-

tion of our results and in particular would explain why only one MC1 protein is strongly bound to the −1 topoisomer.

As measured by ring closure assays (Laine et al.,1991), MC1 has the capacity to facilitate DNA bending. Thermodynamically, such DNA distortions by a protein represent an energetic cost for the binding process. However this energetic barrier is lowered if the DNA is already bent, which explains the higher affinity of MC1 for supercoiled minicircles.

An important result of this study concerns the kinetic aspect of the binding. We show that remarkably stable complexes can be formed with an histone-like protein, even though bacterial chromatin is considered to be a dynamic structure (Drlica & Rouvière-Yaniv, 1987; Gualerzi et al., 1986) where histone-like proteins dissociate rapidly from DNA (Broyles & Pettijohn, 1986; Krzycki et al., 1990). Indeed, even if the half-lives vary with the topological state of the minicircles, they are in any case extremely long for a nonspecific binding. As an example, these complexes are more stable than those obtained in similar conditions with the lactose repressor an archetype of a prokaryotic specific protein (Eismann & Müller-Hill, 1990). Even if the function of MC1 remains elusive, it is likely that it is preferentially bound to DNA loops. One may speculate that MC1 assists specific proteins in building higher order structures via sharp DNA bending.

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