Supercoil-Accelerated DNA Threading Intercalation

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ABSTRACT: The effect of DNA supercoiling on a sterically very demanding threading intercalation process is investigated here. We find that the threading rate of a dimeric ruthenium complex into a negatively supercoiled plasmid at low binding density is 2 orders of magnitude higher than into the cleaved linear form. Further saturation is on the other hand kinetically hampered in comparison to the relaxed DNA. We also observe that threading kinetics correlates with the inhibition of luciferase expression from the plasmid construct. The results show how the target torsional strain can function as a control of DNA threading kinetics and gene expression efficiency.

Topologically constrained DNA is abundant in nature, for instance, as plectonemically supercoiled plasmid DNA or in the form of the toroidal arrangement of chromatin DNA around the histone core in a nucleosome. Whereas torsional strain has a critical role in the compaction of large genomes to fit, e.g., inside a cell nucleus, it also provides a means by which free energy can be stored in the duplex (*1, 2*). For underwound, or negatively supercoiled, DNA this energy can be used to facilitate local opening of the duplex. This has particular implications, for instance, for RNA polymerases which during the initial steps of transcription have to unwind and melt a segment of DNA in the promoter region to access the single-stranded template. Stabilization of this "open complex" by negative supercoiling is believed to be coupled to the enhancement of transcription activation observed with such DNA (*3*−*5*).

Another process associated with the requirement of unwinding and transient strand separation is threading intercalation, by which an intercalating unit, sterically hindered by bulky substituents on both ends, can be inserted between two adjacent DNA base pairs. Antibiotic nogalamycin produced by *Streptomyces nogalater* is a well-known example of a threading agent found in nature (*6, 7*). For the anthracycline moiety to be intercalated, either its polar or nonpolar sugar residue has to pass through the sterically restricted DNA double helix, resulting in slow kinetics. Intercalation of a class of binuclear ruthenium complexes based on the bridging ligand bidppz [for example ΔΔ-**P** (Figure 1a)] is associated with even larger sterical challenges; passage of one large coordinated metal center between the strands is a process requiring large transient distortions of the DNA duplex (*8*−*11*). The energy barrier for unthreading is also high, leading to extremely low exchange rates (*12*), which in turn is considered one of the important characteristics of antigene agents (*6, 13, 14*). The necessity of strand separation has consequences for the preference of binding. ΔΔ-**P** and its analogues are exceptionally selective for alternating A-T stretches longer than 10 bp in oligonucleotide duplexes (*10, 15*). The discrimination was attributed to the higher degree of deformability of such sequences.

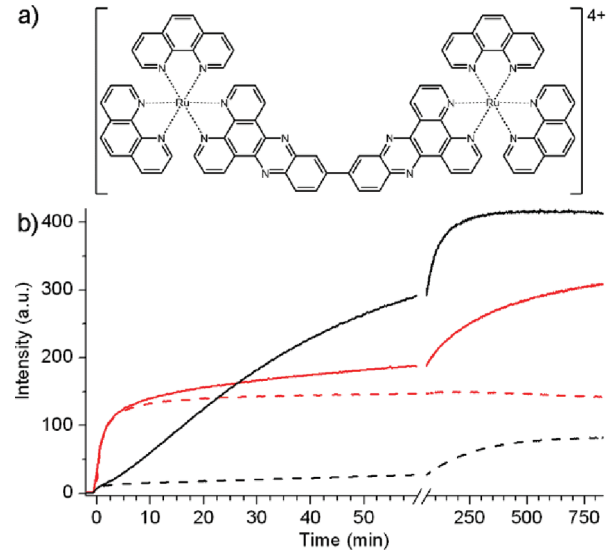

FIGURE 1: (a) Structure of ΔΔ-[μ-(bidppz)(phen)$_4$Ru$_2$]$^{4+}$ [phen is 1,10-phenanthroline, and bidppz is 11,11′-bis(dipyrido[3,2-*a*:2′,3′-*c*]phenazinyl)] (ΔΔ-**P**). (b) Luminescence after mixing ΔΔ-**P** with linearized (black) and supercoiled (red) T7 luc plasmid at 50 °C at a [base pair]/[ΔΔ-**P**] ratio of 16 (—) or 64 (---).

Here we investigate the effect of superhelicity on the threading process by comparing the kinetics of intercalation into luciferase T7 control DNA plasmid (T7 luc, Promega Corp., Madison, WI) in its covalently closed native (negatively supercoiled, $\sigma = -0.09$; see the Supporting Information) and linerarized (relaxed) state. When ΔΔ-**P** is added to mixed sequence calf thymus DNA, a very slow increase in emission intensity is observed as a result of complexes, which initially are groove-bound, rearranging to a luminescent intercalated binding mode (*16*). As expected, the luminescence trace with the relaxed linearized T7 luc plasmid resembles that with calf thymus DNA under similar conditions. At an elevated temperature (50 °C), the binding process, monitored as the increase in luminescence intensity, is completed within a few hours at a [base pair]/[ΔΔ-**P**] ratio of 16 (Figure 1b). Intriguingly, with the closed supercoiled plasmid, the increase in emission intensity follows dramatically different kinetics. The intensity increases rapidly after mixing but is arrested abruptly after 5 min at $^1/_3$ of the final intensity with the relaxed plasmid. In the subsequent phase, the rate of increase is even lower than for the linear form. Thus, the luminescence trace indicates that supercoiling of

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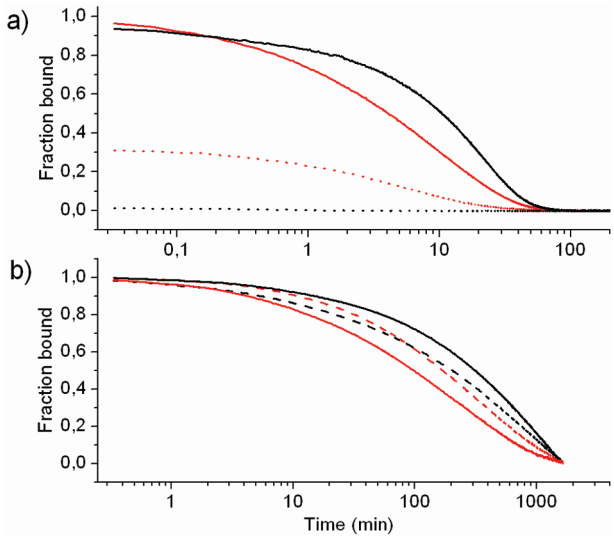

FIGURE 2: Fraction of ΔΔ-**P** intercalated in linearized (black) and supercoiled (red) T7 luc plasmid interpreted from normalized luminescence intensity after addition of a sequestering agent at 50 °C. (a) Addition of SDS 5 min (•••) and 18 h (—) after mixing ΔΔ-**P** and plasmid at a [base pair]/[ΔΔ-**P**] ratio of 16. The intensity prior to addition and in SDS micelles was set to 1 and 0, respectively (corrected for dilution). (b) Addition of poly(dAdT)$_2$ to pre-equilibrated samples at [base pair]/[ΔΔ-**P**] ratios of 16 (—) and 64 (---). Note the different *x*-axis scales.

T7 luc results in biphasic binding; the threading process is drastically facilitated up to a threshold degree of saturation, after which it actually becomes hindered.

Decreasing the amount of added complex to a [base pair]/[ΔΔ-**P**] ratio of 64 results in a roughly proportional decrease in the amplitude of the emission trace with the linear DNA. Contrastingly, for the supercoiled DNA in the initial phase, the traces obtained at high and low mixing ratios overlap (Figure 1b). The emission intensity at low concentrations is thereafter constant, indicating that the reaction is near completion. At an intermediate binding ratio ([base pair]/[complex] ratio of 32), the transition occurs at virtually the same absolute emission intensity, with a subsequent phase with a lower magnitude compared to that for the ratio of 16 (Figure S1 of the Supporting Information). These findings indicate that the initial phase of binding to supercoiled DNA allows roughly one complex per 64 bp to intercalate rapidly.

SDS sequestering, a simple indicator of the fraction of unthreaded complex having a high exchange rate, further supports the findings given above (Figure 2a). When SDS is added after the complex and DNA have been allowed to equilibrate at a [base pair]/[ΔΔ-**P**] ratio of 16, virtually all complexes dissociate slowly from the supercoiled as well as the linear form. At the transition to the slow phase of binding with the supercoiled plasmid (after 5 min at 50 °C), ~30% of the complexes are resistant to rapid sequestering. Consistent with the slow increase in luminescence, virtually all complexes are immediately dissociated from the linearized DNA 5 min after mixing. As SDS is known to catalyze the unthreading process (*12, 17*), sequestering by poly(dAdT)$_2$ better reflects the intrinsic dissociation rate (Figure 2b). The average luminescence quantum yield in the thermodynamically favored alternating AT-DNA is higher than in mixed sequences such as T7 luc (Figure S2 of the Supporting Information), allowing the dissociation process to be easily monitored (*12*). At a high binding density with pre-equilibrated samples, ΔΔ-**P** dissociates faster from the supercoiled than from the linear plasmid ($t_{1/2}$ of 95 and 310 min at 50 °C, respectively), possibly a consequence of a less favorable binding at saturation beyond a [base pair]/[ΔΔ-**P**] ratio of 64. At this low binding density, on the other hand, similar intermediate dissociation rates ($t_{1/2} \sim 165$ min) from the two forms are observed. This is in sharp contrast to the almost 2 order of magnitude difference observed for the association rates.

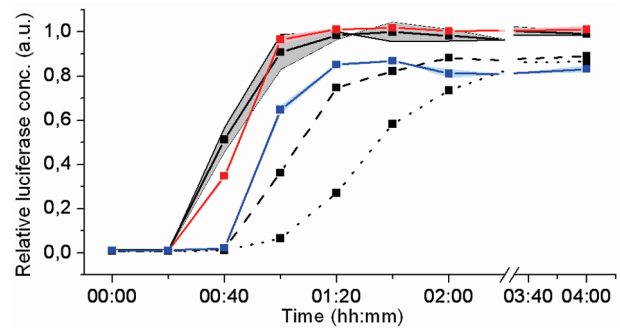

FIGURE 3: Transcription activity determined from luciferase luminescence with time after addition of supercoiled T7 luc template to the reaction mixture. The relative concentration (■) was determined from the mean of two (shaded area) measurements with 0.7 μg of DNA alone (black) or DNA premixed with ΔΔ-**P** at a [base pair]/[complex] ratio of 50 and added either instantly (red) or after incubation for 30 min at 50 °C (blue) to the reaction mixture. Included as references are activity with 0.4 μg of template DNA (---) and activity with the template premixed with actinomycin D at a [base pair]/[ligand] ratio of 50 (•••).

The T7 luc plasmid construct contains a gene encoding luciferase under transcriptional control of a T7 RNA polymerase promoter. With a coupled in vitro transcription/translation system, synthesis of luciferase protein from the template can be quantified from its chemoluminescence. A series of measurements were performed to assess whether threaded ΔΔ-**P** at binding ratios corresponding to the initial rapid phase of binding to supercoiled DNA would interfere with the transcription process. With a rate-limiting amount of plasmid (0.7 μg), the relative luciferase concentration with time was estimated using as a template DNA alone (i), premixed DNA ([base pair]/[ΔΔ-**P**] ratio of 50) directly after mixing (ii), or DNA preincubated for 30 min at 50 °C (iii) (Figure 3). From the kinetic results given above, these conditions are expected to allow complexes under only condition (iii) with supercoiled template to rearrange to the kinetically locked threaded state. The luciferase production of samples (i) and (ii) with supercoiled DNA overlaps well, as expected if the RNA polymerase relatively easily displaces laterally mobile externally bound complexes. The slow kinetics thereby provides an inherent control for effects of the presence of the complex on post-transcriptional steps. After incubation with ΔΔ-**P** at an elevated temperature (iii), we observed a luciferase synthesis that was significantly delayed and reduced, indicating that the threaded state is a greater obstacle for the transcription process. From the concentration profile with a reduced amount of naked DNA (0.4 μg), the inactivation can be estimated to roughly correspond to a 30% reduction in the amount of template available for transcription. As another control, we used actinomycin D, a potent antibiotic known to bind DNA and effectively inhibit RNA synthesis, primarily by interfering with the elongation phase of transcription (*14, 18, 19*).

Indeed, expression from the DNA template with actinomycin D is considerably more inhibited than that with ΔΔ-**P** at the same binding ratio ([base pair]/[ligand] ratio of 50). Increasing the concentration of ΔΔ-**P** is found to only moderately decrease the transcriptional activity (data not shown). With a linearized DNA, no significant effect on the luciferase expression pattern after incubation for 30 min at a [base pair]/[ΔΔ-**P**] ratio of 50 at 50 °C was observed compared to the naked template (Figure S3 of the Supporting Information), again indicative of external binding being ineffective at hindering the RNA polymerase.

In addition to the strong long-range sequence dependence established earlier, we can here present another example of the sensitivity by which a small, but sterically very challenging, ligand can approach its DNA target. Both kinetics and saturation behavior of the threading binuclear ruthenium complex ΔΔ-**P** are shown to intimately depend on the DNA topological strain. At low binding ratios, threading into a circularly closed plasmid at a native superhelix density is ~2 orders of magnitude faster than into its relaxed linearized counterpart. Further saturation appears, on the other hand, kinetically hampered.

An analogy can be drawn to the increased affinity of simple intercalators for supercoiled DNA. The ratio $K_{obs,sc}/K_{obs,lin}$ for ethidium has been reported by Wu et al. to be 1.37 in the limit of low binding density ($\sigma = -0.083$). Duplex unwinding upon intercalation reduces the affinity to a point where the superhelical turns are completely relaxed and the binding constant equals that for the linear form, after which further binding will be at additional expense of binding affinity (*20, 21*).

However, these effects are small compared to the huge difference in threading equilibrium constant (k_{on}/k_{off}) observed here at low binding densities for linear and supercoiled DNA. Furthermore, we find it improbable that the plasmid is completely relaxed at a [base pair]/[ΔΔ-**P**] ratio of 64, since it would require an unwinding angle 8 times higher than that of ethidium (see the Supporting Information). This suggests that other elements of control could be involved in the threading process. We have previously observed that the presence of a loop structure strongly facilitates the threading process in oligonucleotide duplexes, presumably due to the existence of unpaired flexible regions that can act as entrances to the intercalated state (*15*). In relaxed DNA, such alternative structures are relatively rare. However, local energetically unfavorable DNA conformations can be stabilized by the torsional stress of negative supercoiling. Among the most commonly discussed are cruciforms, left-handed DNA and multistranded structures, the occurrence of which is strongly dependent on base pair sequence as well as superhelix density (*1, 2, 22*). The structural heterogeneity of topologically constrained DNA may thus include domains exhibiting lower energy barriers to threading. It is conceivable that such "weak spots" in accordance with the observations could be saturated at a relatively low binding density. Such a property could potentially be utilized to direct complexes to functionally important regions in a designed supercoiled target. The selective threading property becomes particularly interesting with the observation that it correlates with the inhibition of the total RNA synthesis process.

In conclusion, this study points toward a potential of modulating DNA threading efficiency and gene expression pattern by means of target topology. Further investigations of the relation to plasmid sequence and superhelix density are expected to provide more detailed information about the mechanisms of discrimination.

SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures and additional figures and data as mentioned in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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