Kinetics of Site–Site Interactions in Supercoiled DNA with Bent Sequences

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A curved DNA segment is known to adopt a preferred end loop localization in superhelical (sc) DNA and thus may organize the overall conformation of the molecule. Through this process it influences the probability of site juxtaposition. We addressed the effect of a curvature on site–site interactions quantitatively by measuring the kinetics of cross-linking of two biotinylated positions in scDNA by streptavidin. The DNA was biotinylated at either symmetric or asymmetric positions with respect to a curved insert via triplex-forming oligonucleotides (TFOs) modified with biotin. We used a quench-flow device to mix the DNA with the protein and scanning force microscopy to quantify the reaction products. As a measure of the interaction probability, rate constants of cross-linking and local concentrations of one biotinylated site in the vicinity of the other were determined and compared to Monte Carlo simulations for corresponding DNAs. In good agreement with the simulations, a value of 1.74 μM between two sites 500 bp apart was measured for an scDNA without curvature. When a curvature was centered between the sites, the interaction probability increased about twofold over the DNA without curvature, significantly less than expected from the simulations. However, the relative differences of the interaction probabilities due to varied biotin positions with respect to the curvature agreed quantitatively with the theory.

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Keywords: supercoiled DNA; DNA looping; kinetics; DNA curvature; biotin–streptavidin

Introduction

Distant sites on one DNA chain encounter due to internal motions. These motions, and therefore the interaction probability, depend on the DNA structure. Intramolecular interactions are enhanced by supercoiling, which leads to compaction of a circular DNA. The mechanism of site encounter in supercoiled (sc) DNA, reptation along the superhelical axis or global rearrangement of the molecule, depends on the size of the scDNA or the distance between communicating sites. A local DNA curvature centered between interacting loci can facilitate intramolecular contacts. The prokaryotic DNA-bending protein IHF (integration host factor) stimulates the transcription initiation by the upstream-binding activators NifA or NtrC (also denoted as NR) Also, the effect of IHF binding can be replaced by a sequence-directed curvature. It is likely that a curved segment organizes the global conformation of an scDNA by taking a preferred position near an end loop apex of the molecule, an assumption corroborated by scanning force microscopy (SFM) and computer techniques.

Abbreviations used: A-tracts, adenine-tracts; scDNA, supercoiled (superhelical) DNA; strept-POD, streptavidin-peroxidase; TFO, triplex-forming oligonucleotide; SFM, scanning force microscopy. E-mail address of the corresponding author: jl@dkfz-heidelberg.de
Simulations.\textsuperscript{6,17,18} Such a conformation is thermodynamically favored, because the DNA is most pre-bent in the end loops. Then, the constrained end loop localization of the curvature would position two sites that are situated symmetrically relative to the curvature in direct opposition on both superhelical strands, thereby increasing the contact probability. The contact probability of asymmetrically positioned sites should in turn be significantly decreased. However, the strength of such a long-range ordering effect is not yet known. Reduced internal fluctuations of scDNA containing a curved insert have been observed by dynamic light-scattering measurements, consistent with a strong range ordering effect is not yet known. Reduced
decreased. However, the strength of such a long-range positioning sites should in turn be significantly probability. The contact probability of asymmetrically superhelical strands, thereby increasing the contact
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Related to this finding, it has also been observed that the stimulatory effect of the IHF-induced curvature on the transcription activation decreases as the strength of a promoter increases.\textsuperscript{10}

It becomes therefore important to investigate the influence of a local curvature on site-site interactions in scDNA by a direct quantitative measurement. Here, we measured the kinetics of the intramolecular reaction between two specifically biotinylated sites in scDNA by using streptavidin cross-linking in a pulsed quench-flow mixer and SFM to analyze the products. The complex for formation of streptavidin and biotin is irreversible

\[ \text{PS CROSS} \]

\[ \text{P + S} \rightarrow \text{PS + S} \rightarrow \text{PSS} \]

where \( k_1 \) is the rate constant of the binding of free streptavidin to a biotinylated site, two of which are present per scDNA, and \( k_2 \) is the rate constant of the formation of the intramolecular cross-link. Because of the irreversibility of the streptavidin–biotin bond, only the forward reactions are taken into account. Therefore, the formation and subsequent reduction of the intermediate product PS is given by:

\[ \frac{d[PS]}{dt} = k_1[PS][S] - k_2[PS] - k_1[PS][S] \] (1)

The second and third terms in equation (1) describe the formation of the end-products PS\textsuperscript{CROSS} and PSS, respectively, which are written separately in equations (2) and (3):

\[ \frac{d[PS\text{CROSS}]}{dt} = k_2[PS] \] (2)

\[ \frac{d[PSS]}{dt} = k_1[PS][S] \] (3)

According to equation (2) the cross-linking reaction is considered as a first-order reaction: an intramolecular transformation of the intermediate form PS takes place leading to the cross-linked form. At molar excess of streptavidin over the biotinylated DNA, \([S]\) can be considered as approximately constant and equal to the initial concentration \([S]_0\). Under this condition, the formation of PSS is pseudo-first-order and the ratio of the concentrations of the products PS\textsuperscript{CROSS} and PSS is equal to their formation probabilities. Dividing equation
(2) by equation (3) yields:
\[
\frac{d[P_{\text{cross}}]}{d[P_{SS}]} = \frac{k_2}{k_1[S]_0}
\]
which gives upon integration:
\[
\frac{[P_{\text{cross}}]}{[P_{SS}]} = \frac{k_2}{k_1[S]_0}
\]

Thus, the ratio of cross-linked to double bound products is independent of the concentration of the intermediate PS and constant in time. This means, that \(k_2\) can be obtained by observing the ratio \([P_{\text{cross}}]/[P_{SS}]\) from equilibrium reactions, if the rate \(k_1\) is known.

An important aim of this study was to compare kinetic data for the cross-linking reaction with Monte Carlo simulations, by which \(j_M\) can be calculated. \(j_M\) measures the probability that one site on the DNA is found in a sphere with given radius \(r\) around a second site, which is obtained from the distribution of DNA conformations in thermodynamic equilibrium.\(^{23,24}\) \(j_M\) is approximated here by considering the cross-linking reaction as an internal bimolecular reaction. By assuming that the “internal rate constant” of this reaction is equal to \(k_1\), the cross-link formation is given by:
\[
\frac{d[P_{\text{cross}}]}{dt} = k_1[P_{SS}]_0 \cdot j_{M,\text{app}}
\]
where \(j_{M,\text{app}}\) is the apparent local concentration of one reactive group in the vicinity of the other. Corresponding to the steps in equations (4) and (5), dividing equation (6) by equation (3) yields:
\[
\frac{[P_{\text{cross}}]}{[P_{SS}]} = \frac{j_{M,\text{app}}}{[S]_0}
\]

We assume that equations (6) and (7) apply, because the conformational equilibration of the DNA is fast enough that the distribution of DNA conformations present at the moment of streptavidin addition determines the rate of cross-linking. However, a noteworthy source of error by the derivation of \(j_M\) in this way may be that the internal rate constant may be changed depending on structural or dynamic properties of the DNA, as will be discussed below in detail. Additionally, it is critical that biotin binding to the streptavidin tetramer is not cooperative: this has been demonstrated by incubating biotin saturated with biotin-free streptavidins, where after several days a uniform distribution of biotinylation levels was obtained.\(^{25}\)

### Properties of the analyzed scDNAs

The analyzed scDNAs had similar superhelical densities \(\sigma\), which were typical for plasmids isolated from *Escherichia coli*, as determined by band counting.\(^{26}\) Only pUC18-4A-H1/2, the plasmid containing the curvature type with A-tracts, had a slightly lower \(\sigma\)-value compared to the other DNAs (Table 1).

The intrinsic curvature of the entire plasmid sequences was calculated to demonstrate the change in the DNA conformation due to the curved insert. The equilibrium conformation of the sequences, as calculated with the program Curvature,\(^{27}\) can be seen in Figure 1. The strong curvature of the A-tracts containing region is

### Table 1. Properties of the examined plasmid DNAs

<table>
<thead>
<tr>
<th>Plasmid (pUC18)</th>
<th>Size (bp)</th>
<th>Distances between each label and the center of curvature (bp)</th>
<th>Superhelical density, (-\sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/6A</td>
<td>2571</td>
<td>304/305</td>
<td>0.057</td>
</tr>
<tr>
<td>H1/4-1</td>
<td>2521</td>
<td>304/255</td>
<td>0.058</td>
</tr>
<tr>
<td>5/6A</td>
<td>2522</td>
<td>304/220</td>
<td>0.056</td>
</tr>
<tr>
<td>H1/2</td>
<td>2535</td>
<td>309/228</td>
<td>0.045</td>
</tr>
<tr>
<td>4A</td>
<td>2491</td>
<td>501 (uncurved)</td>
<td>0.057</td>
</tr>
</tbody>
</table>

*Figure 1. Equilibrium conformation of the examined DNA sequences. The DNA conformations were calculated with the program Curvature\(^{27}\) on the basis of the algorithm of Bolshoy et al.\(^{42}\) The plots show the linearized plasmids with one bead corresponding to 21 bp. The region that is expected to be curved due the presence of the phased A-tracts is colored red. Only in the DNA without phased A-tracts, pUC18-1A-H1/2, is the corresponding region straight.*
clearly distinguishable but other more weakly bent regions exist. The bending angle of the A5–6-insert can be assumed to be at least $108^\circ$, on the basis of experimental findings.\textsuperscript{28–30}

**Site-specific labeling of scDNA**

It has already been demonstrated that scDNA can be site-specifically labeled by triple helix formation with TFOs.\textsuperscript{22} Here, the labeled DNAs contained two TFO-binding sites of different sequences. The specific hybridization of the TFOs to these binding sites was verified by gel shift analysis (Figure 2). Incubation with only one TFO resulted in a gel shift of only the fragment that contains the corresponding binding site, indicating that cross-reaction is negligible. Incubation with both TFOs yields double-labeled DNA. We obtained a labeling efficiency of 70–95% at each TFO-recognition site, which was sufficient for the purpose of the measurements.

The label is bound covalently to the target DNA by a 5'-psoralen modification of the TFOs, since pyrimidine-purine-pyrimidine triplexes are stable only at acidic pH values.\textsuperscript{22,31} Since all reactions were near neutral pH, we assumed that the TFOs were able to move freely around the covalent DNA–TFO connection via the psoralen group, and that this connection site defines the exact positions of the labels. The resulting estimated distances between the biotin molecules and the center of the curvature in all analyzed DNAs are listed in Table 1.

**Rate constant $k_1$ of the binding of free streptavidin-peroxidase to one biotinylated site**

The rate constant $k_1$ has to be known to determine the rate of cross-linking $k_2$ (equation (5)) and was therefore measured using single-biotinylated DNA. For all measurements, we used a 140 kDa streptavidin-peroxidase conjugate (strept-POD) because this can be visualized by SFM. First, the concentration of active strept-POD in our stock solution was determined by titrating single-biotinylated DNA with the protein (Figure 3(a) and (b)). By SFM imaging of an accidentally chosen sample from the initial linear increase of the binding curve (Figure 3(b)), we observed that not all of the four binding sites of strept-POD were occupied by biotinylated DNA, which may be explained by steric hindrance. A distribution of monomers (37.6%), dimers (55.9%), trimers (6.5%) and no tetramers was found, yielding the binding stoichiometry (not shown).

The determined activity of the protein was used to specify the conditions for the measurement of $k_1$. In Figure 4, two kinetics are plotted that have been measured using a quench-flow apparatus at strept-POD concentrations of either 60 nM or 80 nM. The protein was at a 13 or 18-fold excess over the biotinylated DNA, so that pseudo-first-order reactions were assumed. Fitting of a single exponential to the observed data points yielded an average molar rate constant of $k_1 = 5.3(\pm 0.3) \times 10^{-7} \text{ M}^{-1} \text{s}^{-1}$. In these reactions, a binding stoichiometry of one strept-POD per one biotinylated DNA had to be assumed, in contrast to the titration samples. Almost no intermolecularly linked DNAs were formed in the reactions with double-labeled DNA described in the next section, which were conducted also in the quench-flow apparatus at the same DNA concentration as the quenched reactions. Moreover, one sample of the initial phase of a kinetic was analyzed by SFM and no DNAs interlinked by one strept-POD were found (not shown).

**Measurements of the intramolecular cross-linking reaction**

The set of scDNAs characterized in Table 1 was used to examine the effect of a curved insert on the cross-linking reaction. The cross-linking ratio $[\text{PSCROSS}] / [\text{PSS}]$ was determined by mixing scDNA biotinylated at two sites with strept-POD in the quench-flow mixer followed by SFM analysis. Figure 5(a) illustrates the strategy to detect the reaction products on SFM images by cleavage with \textit{Pvu}II restriction endonuclease. The corresponding SFM micrographs in Figure 5(b) show single molecules representing the complexes PSCROSS and PSS. For each sample, 63–278 molecules were counted. On four plasmids the reactions were conducted at different concentrations.

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**Figure 2.** Detection of site-specific triplex formation. Binding of the TFOs (3 and 4) to pUC18-5/6A-H1/2 is shown in a 6% polyacrylamide gel after digestion of the DNA with \textit{Hae}III restriction endonuclease. The recognition site of TFO 3 resides within the 616 bp fragment, and that of TFO 4 within the 232 bp fragment of the digest. The lanes contain a length standard (L), a control DNA (C) and DNA with bound TFO 3 (1), TFO 4 (2) and both TFOs (3).
It can be seen in Figure 6 that the cross-linking ratios are increased in all curvature-containing plasmids as compared to the DNA without curvature, pUC18-1A-H1/2. The most significant increase was observed, as expected, in the plasmid pUC18-5/6-H1/4-I with symmetric biotin positions with respect to the A₅–₆-curvature. This corresponded to a 2.1-fold increase in the intramolecular rate constant $k_2$ and the apparent local concentration $j_{app}M$:

Defining the degree of asymmetry $\Delta s$ as the difference in the distances between the two biotin molecules and the center of curvature, the interaction probability decreased systematically with increasing asymmetry to a value at $\Delta s = 84$ bp (pUC18-5/6A-H1/2), only slightly higher than in the DNA without curvature. In the plasmid with the $A_4$-insert ($\Delta s = 81$ bp) the interaction probability was similar to that of the plasmid with the stronger bent $A_{s-o}$-curvature and corresponding $\Delta s$ (84 bp), indicating that neither a smaller superhelical density (Table 1) nor a smaller bending angle reduces the interaction probability. One sample of the uncurved DNA was analyzed in its open circular form and showed a significantly lower interaction probability than the supercoiled counterpart (6.4-fold). For this sample, however, we obtained only one datum point, so that $k_2$ and $j_{app}M$ have not been calculated by fitting. The $k_2$ and $j_{app}M$ values for all examined plasmids are summarized in Table 2.

**Figure 3.** Binding activity of strept-POD. (a) A native 8% polyacrylamide gel electrophoresis of DNA, which was biotinylated at the TFO-binding site H1, incubated with strept-POD and then HaeIII-digested (triplex-formation at H1 is within the 616 bp fragment of the digest, see Figure 2). Lane 1, length standard; lane 2, biotinylated plasmid; lanes 3–8, incubation with incremental amounts of strept-POD. (b) Quantitative image analysis. The binding curve observed from two individual measurements indicated as filled circles and triangles, respectively, is shown (the triangles correspond to (a)). The plot shows the percentage of DNA bound by strept-POD (%PS) versus the amount of added strept-POD. The plateau indicates the proportion of TFOs carrying active biotin. The open circle shows the amount of bound DNA determined on SFM images. The inset is shown to demonstrate the initial linear increase of binding.

**Figure 4.** Quench-flow kinetics of strept-POD and single-biotinylated DNA. Two kinetics at strept-POD concentrations of 60 nM (○) and 80 nM (△) are shown. Percentages of DNA complexed with strept-POD (%PS), as analyzed by electrophoresis corresponding to Figure 3(a), are plotted versus the reaction time. The data were fitted with a single-exponential function (straight lines).
Simulations of the interaction probability

Table 2 also shows simulated \( j_M \) values for the analyzed DNAs at different reaction radii \( r \). A strong enhancement of \( j_M \) over the DNA without curvature is observed for all scDNAs with curvature, in agreement with previous calculations performed with the same computer program.\(^{17}\) Both the absolute values and the relative differences between the DNAs are dependent on \( r \). For example, for the DNA with curvature and symmetrical sites (\( \Delta s = 1 \) bp) a 9.6 and a 6.5-fold increase of \( j_M \) over the DNA without curvature was calculated at reaction radii of 10 nm and 20 nm, respectively. \( j_M \) decreases with increasing asymmetry \( \Delta s \), as observed in the experiment. We did not take into account the variation in bending angle between the two curvature-inducing sequences, since the adopted 100° represents most likely a slight underestimate for both sequence types. In the earlier simulations, changes of the bend angle affected \( j_M \) only below 100° at the separation between sites comparable to the present study.\(^{17}\) The slightly lower simulated value for the DNA with the A_4-insert compared to the DNA with the A_5–6-insert and corresponding asymmetry is therefore due to the lower superhelical density (see Table 1).

Comparison of experimental and simulated results

Since \( j_M \) is strongly dependent on the reaction radius \( r \), we estimated this parameter for the cross-linking reaction as follows: the TFOs are expected to move freely around the covalently bound psoralen group. Therefore, the reaction radius is twice the average end-to-end distance (\( \langle R \rangle \)) of the TFOs. By regarding a TFO as a flexible chain, \( \langle R \rangle \) can be estimated from the relation \( \langle R^2 \rangle = 2L_cL_o \) where \( L_o \) is the persistence length and \( L_c \) is the contour length of the chain. Assuming a rise per nucleotide residue of 0.6 nm and a persistence length of 2 nm of single-stranded DNA,\(^{32,33}\) the calculation of \( \langle R \rangle \) yields 7.7 nm or 8 nm for the TFOs comprising 25 or 27 nucleotide residues, respectively. Adding the size of streptavidin (about 5 nm, estimated on the basis of the partial specific volume of the protein using the program Sedenterp (version 1.05)\(^{34}\)), the reaction should take place with highest probability at a spacing between the TFO connection sites in the range of 15–20 nm. Comparing the experimental and the simulated results, we observe the following:

1. For the DNA without curvature the measured apparent local concentration \( j_M^{app} \) of 1.74 µM
lies between the simulated \(j_M\) values at reaction radii of 15 nm and 20 nm (2.6 \(\mu\)M and 1.4 \(\mu\)M).

2. The measured and the simulated relative differences of the interaction probabilities as a function of the degree of asymmetry \(D_s\) of the labeling sites with respect to the curvature are also in quantitative agreement for the estimated range of reaction radii. This is illustrated in Figure 7.

3. The measured absolute interaction probabilities of all DNAs with curvature are significantly less than predicted by the theory.

**Discussion**

**Time-scale of site–site interactions in scDNA**

A number of studies have analyzed the kinetics of site–site interactions in scDNA from a theoretical point of view. From Brownian dynamics simulations one obtains first collision times \(\tau_F\) between two sites in the range of 1–3 ms for DNAs comparable to the DNA without curvature of the present study.\(^7\) For this DNA the average reaction time \(\tau = 1/k_2\) of the cross-linking reaction is 10.8 ms. For a reasonable comparison of the measured and the simulated reaction times one has to consider the internal rate constant of the interacting groups, which we approximate by the measured rate constant \(k_1\) between strept-POD and a single biotinylated site on the DNA \((5.3 \pm 0.3 \times 10^{-7} \text{M}^{-1} \text{s}^{-1})\). If this value is below the diffusion-controlled limit, a time \(\tau_R\) from the first collision to the final reaction should contribute to the reaction time of cross-linking. Both, \(\tau_F\) and \(\tau_R\) can, of course, not be distinguished by the analyzed reaction system. Yet, simulations of irreversible site–site interactions for different internal rate constants yield an average total reaction time \(\tau_S = \tau_F + \tau_R\) of 8.0 ms for conditions comparable to the

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**Figure 6.** Cross-linking ratio as a function of the concentration of strept-POD. The cross-linking ratios \([\text{PS}]/[\text{PSS}]\) for increasing strept-POD concentrations are shown, as determined by counting on SFM images. The plots each display the uncurved control, pUC18-1A-H1/2 (○) versus one curvature containing plasmid: (a) pUC18-4A-H1/2 (■); (b) pUC18-5/6A-H1/2 (▼); (c) pUC18-5/6A-H1/4 (▲); and (d) pUC18-5/6A-H1/4-I (●). The lines represent fitting of equations (5) or (7) to the data points. Only for pUC18-5/6A-H1/4-I, the cross-linking ratio was determined four times at one strept-POD concentration.
cross-linking reaction. This result agrees well with the measured value, and in that case, a first collision time of 3 ms was calculated.

The kinetics of intramolecular interactions in scDNA have been investigated experimentally using the resolvase system of site-specific recombination. The recombination process, which requires synapsis of two bound resolvases, covered a large period of time (10 ms–100 seconds), as analyzed using a quench-flow apparatus. Subsequent computer simulations suggested that this was due to the formation and rearrangement of non-productive synapatic complexes caused by certain unfavorable scDNA conformations. Applying this hypothesis, the short time-scale of the cross-linking reaction is comparable to the resolvase system, inasmuch that all DNA conformations with the biotinylated sites located in close proximity to each other should, in principle, lead to the reaction. Approach between the sites may occur due to both the slithering motion of the DNA strand and bending of the superhelix axis.

Influence of a curvature on site–site interactions

A quantitative comparison of the experimental results with the Monte Carlo simulations allows us to verify the predicted long-range effect of a curvature located in an end loop. For the estimated range of reaction radii of 15–20 nm the simulations match the measured relative differences due to varied biotin positions with respect to the A5–6 curvature. This result can be interpreted by a strong organizing effect of the curvature on the conformation of the examined scDNAs. The fact that the theory agrees with the experiment for the DNA without curvature at the same range of reaction radii corroborates this conclusion. On the other hand, the absolute increase of the measured interaction probabilities in all scDNAs with curvature over the DNA without curvature is substantially lower than what was expected on the basis of the simulations. We assume that one major conceptual difference between the experiment and the simulation can explain this deviation: in the experiment a protein bound to a TFO participates in the cross-linking reaction, whereas the simulation measures only the approach of certain positions on the DNA strand. The diameter of the hydrated strept-POD-conjugate of about 8 nm (calculated with the program Sedenterp, version 1.05) is in the range of the average distance between the opposing DNA strands in the superhelix at the given salt conditions. Therefore, a free movement of the initially bound strept-POD around the DNA strand may be decreased, thereby reducing the accessibility of the second biotin molecule for the bound strept-POD. This explanation would imply that the accessibility is reduced only in scDNA with curvature and negligible in scDNA without curvature. This hypothesis may be rationalized by reduced internal dynamics of scDNA with curvature, a view that our earlier observations support; namely, that an A-tract-induced curvature reduces the amplitude of the internal motions of scDNA. Nevertheless, the simulated $j_M$ values correctly reflect the relative differences of the cross-linking rates among the plasmids with $A_{5–6}$ curvature and varied biotin positions, since each

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**Table 2. Intramolecular rate constants $k_2$ and local concentrations $j_M$**

<table>
<thead>
<tr>
<th>Plasmid (pUC18)</th>
<th>$\Delta s$ (bp)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$j_M^{exp}$ (µM)</th>
<th>$j_M$ (µM), simulated for $r = 10, 15,$ and 20 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>$-5'/6'A-$H1/4</td>
<td>1</td>
<td>192.8 (± 11.3)</td>
<td>5.63 (± 0.21)</td>
<td>42.2, 21.6, 9.10</td>
</tr>
<tr>
<td>$-5'/6'A-$H1/4</td>
<td>49</td>
<td>174.6 (± 3.9)</td>
<td>3.30 (± 0.07)</td>
<td>30.5, 18.0, 8.80</td>
</tr>
<tr>
<td>$-5'/6'A-$H1/4</td>
<td>84</td>
<td>115.1 (± 11.2)</td>
<td>2.17 (± 0.20)</td>
<td>16.1, 11.7, 7.60</td>
</tr>
<tr>
<td>$-4A-H1/2$</td>
<td>81</td>
<td>117.1 (± 10.0)</td>
<td>2.20 (± 0.18)</td>
<td>12.1, 9.80, 6.60</td>
</tr>
<tr>
<td>$-1A-H1/2$</td>
<td>4</td>
<td>92.3 (± 8.9)</td>
<td>1.74 (± 0.25)</td>
<td>4.40, 2.60, 1.40</td>
</tr>
<tr>
<td>$-1A-H1/2$</td>
<td>14.2 (± 2.3)</td>
<td>0.27 (± 0.04)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values for $k_2$ and $j_M^{exp}$ were calculated by fitting equations (5) and (7), respectively, to the cross-linking ratios $[P_{SCROSS}]/[P_{SS}]$. Only for pUC18-5'/6'A-H1/4, for which the $k_2$ and $j_M^{exp}$ were calculated directly and averaged.

* $\Delta s$: Difference of the distances between labels and the center of curvature (see Table 1).

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**Figure 7. Effect of the degree of asymmetry $\Delta s$ on the local concentration $j_M$.** The $j_M$ values are normalized with respect to the value for symmetrically positioned sites ($\Delta s = 1$ bp). The plot shows the measured ($\bullet$) and the simulated (reaction radii of 10 nm ($\blacksquare$), 15 nm ($\blacktriangle$) and 20 nm ($\blacktriangleup$)) values.
of these plasmids underlies the same steric effect. Changes of the internal dynamics of scDNA due to the curvature might influence the internal reactivity significantly, since the diameter of strept-POD and the interstrand distance in the superhelix are approximately of the same size. A weak steric hindrance may also apply for scDNA without curvature, since the measured 6.4-fold increase in $j_{\text{app}}$ compared to the relaxed form is, albeit significant, less than what has been predicted by Monte Carlo simulations.5

In addition, the extent of such a steric effect may be associated with the mechanism of a site–site interaction. Steric hindrance of a bound protein should be pronouncedly stronger if interactions would take place by an alignment of the sites opposite to each other in the superhelix rather than by bending of the superhelix axis. The constrained end loop position of the curvature can be expected to enforce the first mechanism, which has also been observed by Brownian dynamics simulations.8

Sequence-specific properties of the entire scDNAs, which are not taken into account in the simulations, may have contributed to the deviation between experiment and theory. A calculation of the intrinsic curvature of the complete plasmid sequences shows that several regions are weakly bent, in addition to the phased A-tracts (Figure 1). These may affect the equilibrium conformation and the mode of conformational changes of the molecule.6 However, SFM measurements of the same site-specifically labeled scDNAs did not reveal any preferred DNA conformations except for the end loop position of the curved insert.15 Therefore, we assume that the plasmid sequence outside the A-tracts plays only a minor role.

Comparison of different curvature-inducing sequences

The difference in bending angle of both examined curved sequence types did not affect the interaction probability. Also previous Monte Carlo simulations suggest no significant influence on $j_{\text{app}}$ for different angles above 100°.17 However, the simulations cannot account for possible structural changes in A-tract sequences due to supercoiling. Reported experimental results on the dependence of the end loop localization on the bending angle are contradictory: we observed by SFM measurements that the $A_{0/1}$-insert exhibits a higher probability of the end loop localization than the $A_{1}$-insert, apparently consistent with a higher-bending angle of the $A_{0/1}$-insert.15,21 In contrast, Tsen & Levene68 showed in a site-specific recombination assay that also an intrinsically straight insert with A-tracts out-of-phase exhibits the preferred end loop position, which the authors attributed to an increased flexibility of A-tracts as a result of supercoiling. The present result suggests that differences in the interaction probability due to altering the sequence composition of the curved insert are rather small and can therefore not be resolved by this reaction system.

Conclusions

For scDNA without curvature, the measured interaction probability as given by the apparent local concentration $j_{\text{app}}$ agrees well with the Monte Carlo simulation, which also confirms previous theoretical studies.8,17 Also a good agreement with Brownian dynamics simulations was observed with regard to the measured rate constant of cross-linking in the DNA without curvature, indicating a millisecond time-range for the kinetics of site–site interactions in scDNA. Furthermore, our work demonstrates a significant influence of a curvature on the interaction probability, although the measured absolute cross-linking rates were not completely reflected by the simulations. We conclude that a curvature can enhance $j_{\text{app}}$ between symmetric sites in scDNA over a long distance. The deviation between the absolute $j_{\text{app}}$ values in theory and experiment might suggest that the intramolecular interaction is sensitive to changed dynamics of scDNA due to a curvature.

Material and Methods

Studied DNAs

The measurements were done on the derivatives of pUC18; pUC18-1A-H1/2, pUC18-4A-H1/2, pUC18-5/6A-H1/2, pUC18-5/6A-H1/4 and pUC18-5/6A-H1/4-I. These DNAs are similar to those already described,18 except for an insertion of a second 31 bp (H2) or 30 bp (H4) homopurine-homopyrimidine (pu/py) segment. All insertions denoted as $H^X$ (X = 1, 2 or 4) are binding sites for triplex-forming oligonucleotides (TFOs). The segment H2 (5’-CTTCCTCTCCTCTCTCCTCTCTCTCTCTCTCTCTCAG-3’) was cloned into the single EcoRV site and the segment H4 (5’-TTATAGAAAGAGAGAGAGAGAGGAAG-3’) into the single SapI site of the respective parent plasmids. Furthermore, a 48 bp fragment (I) obtained by cleavage of the plasmid pK-4A108 with HindIII and EcoRI, and treated with Klenow enzyme to produce blunt ends was inserted into the EcoRV site of pUC18-5/6A-H1/4 to obtain the plasmid pUC18-5/6A-H1/4-I. The orientation of the inserted fragments within the DNAs was verified by sequencing using the dideoxy-termination method. The notations 4A- and 5/6A- refer to inserts, which exhibit sequence-induced curvature due to repetitive adenine tracts located in phase with the helical turns.21 As a control, pUC18-1A-H1/2 with a non-curved insert (+1A) was examined. The curved inserts have total lengths of 80 bp (A-type) and 67 bp (A-type). The cloned plasmids containing the $A_{0/1}$-insert, the stronger bent sequence,21 were designed in such a way that symmetric and two different asymmetric label positions with respect to the curvature could be investigated. In the plasmid containing the $A_{1}$-insert the labeling sites were located asymmetrically (Table 1).

After amplification in E. coli Nova Blue cells, the plasmids were isolated using the Plasmid Maxi Kit (QIAGEN, Hilden, Germany) and purified by HPLC.
using a reverse phase column (RP-18, Merck, Germany) to remove linear and open circle (oc) DNA species. The >90% supercoiled (sc) DNA obtained by this purification method was analyzed by the band-counting method to determine the average linking number (ΔLk) of the plasmids. The sequence-dependent curvature of the complete plasmid sequences was analyzed using the program Curvature on the basis of the algorithm derived by Bolshoy et al.

**Site-specific biotinylation**

The superhelical DNA was labeled by TFOs which are 3′-modified with biotin and 5′-modified with C6-psoralen (Figure 2). Three homopyrimidine-TFOs are designed such that they can hybridize to the pu-py sequences (HX, X = 1, 2 and 4), two of which are present in the analyzed plasmid DNAs. TFO 3 binds to the sequence H1 and TFO 4 (5′-TCTCTTCTCTTCTCTCTCTCTCTCCTC-3′) and TFO 6 (5′-TCTCTTCTCTTCTCTCTCTCTCCTC-3′) bind to the sequences H2 and H4, respectively. The oligonucleotides were manufactured by Eurogentec (Seraing, Belgium). Labeling solutions contained 200 nM plasmid-DNA in 10 mM sodium acetate, 50 mM MgCl2 (pH 5.0), and 5 μM each TFO. They were incubated at 70°C for ten minutes and then cooled slowly to room temperature. By photo-cross-linking at 350 nm for two minutes, irradiating through the monochromator of an SLM-AMICO 8100 fluorescence spectrometer (SLM, Urbana, IL), the psoralen was bound covalently to the target DNA. The unbound TFOs were then removed by ethanol-precipitation or by gel-filtration using a Superose 6-column (Pharmacia, Freiburg, Germany).

Analysis by Hae III digestion and subsequent electrophoresis on a native 6% (w/v) polyacrylamide gel showed the binding of the TFOs to the respective target sites. TFO binding results in a characteristic band shift of the fragment containing the recognition site (Figure 2). After electrophoresis, the gel was stained with ethidium and the labeling yield was determined by band quantification using the program BioImage (version 2.1.1, B.I. Systems Corp., USA).

**Binding activity of streptavidin-peroxidase**

For all measurements described in the following a 140 kDa strept-POD was used, which could be clearly visualized by SFM imaging. To analyze the binding activity of strept-POD with respect to biotinylated DNA we incubated 0.6 pmol of DNA single-labeled at the pu-py-sequence H1 with varying amounts of strept-POD in 5 mM magnesium acetate, 50 mM ammonium acetate, 0.1 mM EDTA, 5.0 mM Tris–HCl (pH 7.2). The concentration of the strept-POD stock solution was as specified by the manufacturer (7 pmol/μl, Pierce, Rockford, USA). The DNA concentration was determined from its absorbance at 260 nm in a Cary-4E spectrometer (Varian, Mulgrave, Australia). After one hour of incubation, biotin in molar excess was added. The DNA was then Hae III-digested and run on a native 8% polyacrylamide gel. The amount of DNA bound to strept-POD was quantified by means of the shifted 616 bp Hae III fragment, to which the TFO is attached, and which is further shifted due to strept-POD-binding. A control not incubated with strept-POD was run on the gel in order to determine the amount of TFO binding. Band intensities were quantified as mentioned above. The proportion \( p \) of the band-intensity of the TFO bound fragment (the 161 bp-band) relative to one other band was calculated. The percentage of the \( p \)-value of each sample relative to the \( p \)-value of the control equals the amount of unbound biotinylated DNA, which was then converted to the amount of bound DNA.

The binding stoichiometry was determined by SFM (see below) of one titration sample, in which the DNA was in molar excess. Single-labeled scDNAs intermolecularly linked by strept-POD can be identified on SFM images so that monomers, dimers, trimers and tetramers can be distinguished (compare with the intramolecular cross-links in Figure 5(b)). The ratio \( i \) of active strept-POD per bound biotinylated DNA is then given by \( i = f_{\text{monomers}} + f_{\text{dimers}}/2 + f_{\text{trimers}}/3 + f_{\text{tetramers}}/4 \), where \( f_i \) is the frequency of the respective complexes. The amount of the effectively biotinylated DNA \( P_{\text{biot}} \) is given by \( P_{\text{biot}} = P_{\text{tot}} P_{\text{biot}}/P_{\text{nuc}} \), where \( P_{\text{biot}} \) is the total amount of DNA, \( P_{\text{nuc}} \) is the proportion of DNA with bound TFO and \( P_{\text{tot}} \) is the proportion of TFOs carrying active biotin. The latter is given by the plateau of the titration curve. Extrapolation of the initial linear increase of the curve to the plateau yielded the point at which the amount of biotinylated DNA times the determined ratio \( i \) equals the amount of added active strept-POD.

**Quench-flow reactions**

A quench-flow device (QFM-5, Biologic, France, see Figure 8) was used for the measurement of the rate constant \( k_i \) of the binding of strept-POD to single-biotinylated DNA. Approximately 4.5 nM (final concentration) single-labeled plasmid-DNA driven from syringe II was mixed with strept-POD (Pierce, Rockford, USA) at a final concentration of either 60 nM or 80 nM from syringe III. The reactions took place in 5 mM magnesium acetate, 50 mM ammonium acetate, 0.1 mM EDTA, 5.0 mM Tris–HCl (pH 7.2). The flow rate was set to 2 μl/ms and a delay-line II of 100 μl (117 μl effective volume between the mixers) was used. After varying time delays from 0 ms to 2000 ms the reaction solution was mixed with 35 μl of the quench solution from

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**Figure 8.** Schematic representation of the quench-flow module QFM-5. Solutions can be driven from four syringes (S 1–4) into the capillary system composed of the mixers M 1–3 and the reaction chambers, i.e. the delay lines DI1 and 2. C, collection port; S 5, fifth syringe by which aliquots of the reaction mixture can be accommodated that are to be discarded. The syringes are driven by stepping motors, movements of which are controlled on a personal computer (Stopped-Flow-Module-Application-Driving-Software, version 1.15, Biologic, Claix, France).
syringe 4 containing about 200 μM free biotin. By driving the reaction mixture with water from syringe 1 out of the reaction chamber, a 135 μl aliquot of the total reaction volume was collected. Reactions were at 26 °C. The reaction products were quantified corresponding to the titration experiment (see above).

To examine the intramolecular cross-linking reaction, the quench-flow device was used to mix double-biotinylated DNA with strept-POD, but without quenching the reactions. Here, the instrument was used as a mixing device, since (i) it allows optimal mixing of the reactants, so that no local inhomogeneities of the streptavidin concentration could have affected the cross-linking ratio and (ii) the reaction system was calibrated with respect to $k_1$. All settings of the instrument were as described for the measurement of $k_1$, except that no quench solution was added. The reactions took place at 26 °C in 75 mM NaCl, 5.0 mM NaP$_i$, 0.1 mM EDTA, 5 mM Tris–HCl (pH 7.15). Strept-POD concentrations ranged from 0.16 μM to 0.48 μM. A control experiment (not shown) confirmed that the plasmid-DNA was not nicked by shearing at flow rates from 0.5 ml/ms to 4 ml/ms.

### Scanning force microscopy

The reaction products from the equilibrium reactions with double-biotinylated DNA were visualized by SFM to determine the proportion of cross-linked plasmids (PSt-POD) and those which had bound two strept-PODs (PSS) by counting. The counting error was estimated assuming the standard deviation of a binomial distribution. An advantage of this quantification method is that reaction products of double-biotinylated DNAs can be distinguished from those of single-biotinylated DNAs, so that a correction for the biotinylation efficiency was not necessary. The reaction products were digested with PvuII-restriction endonuclease (see Figure 5), and subsequently the majority of unbound strept-POD and restriction endonuclease was removed by ethanol-precipitation (sample redissolved in 10 mM Tris (pH 7.5), 0.1 mM EDTA).

For the scanning process the samples were prepared by the magnesium-mediated adsorption technique. A total of 1.5 μl of the DNA was mixed with 20 μl of a deposition buffer (10 mM Hepes (pH 8.0), 100 mM MgCl$_2$, freshly added 0.01% (v/v) Nonidet P-40). The solution was dropped onto freshly cleaved mica, which was then immediately washed with 50 ml of Millipore-purged water and finally dried in a nitrogen stream. The SFM images were generated with a Nanoscope III microscope (Digital Instruments Inc., Santa Barbara, CA) using etched silicon tips (L. O. T. Oriel, Darmstadt, Germany) in “tapping mode” with a drive frequency of 300–400 kHz. The scan diameter was 3–5 μm$^2$ with 512 x 512 pixels per image.

### Computer simulations

The simulations were performed as described previously, except for some parameters that account for the experimental conditions of this study. In brief, the scDNA is considered as an elastic rod consisting of $n_k$ elementary segments, where $n$ is the number of Kuhn segment lengths (100 nm) and $k$ is the number of segments per Kuhn length. Three parameters characterize the rod: the torsional and the bending rigidity constants and the effective diameter of the DNA, which depends on the salt conditions in the medium. A permanent bend of angle $\Theta$ within the chain is regarded in the DNA model by setting bends of angle $\Theta/j$ at $j$ joints between successive segments. Using these parameters, an ensemble of DNA conformations is generated at thermodynamic equilibrium by the Monte Carlo procedure.

The contour length $L$ was set equal to that of the DNAs analyzed experimentally ($L = 850–875$ nm with $N = 170–175$ elementary segments, respectively). The linking number deficit $\Delta Lk$ corresponded to the average linking number deficit determined for the analyzed DNAs (see Table 1) and the torsional rigidity was set to $2.5 \times 10^{-19}$ erg cm. For the effective chain diameter a value of 2.4 nm was adopted, corresponding to an ionic strength of 85 mM. For all simulated DNAs with curvature a bending angle of the A-tract sequence of $\Theta = 100^\circ$ was adopted.

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### References


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