Chapter 2
Methods used in the oxidation of DNA

Studying the oxidation of DNA requires a variety of methods and techniques in order to evaluate the DNA cleaving properties of catalysts. In this chapter, the selection of plasmid DNA as a benchmark substrate is discussed as this type of DNA allows for analysis of the DNA cleavage process by gel electrophoresis during and after reaction. These results can be placed in context using appropriate statistical analysis methods, which allows for the discrimination between single strand DNA cleavage and (direct) double strand DNA cleavage. Furthermore, new methods, which can be used in examining the DNA cleaving properties of catalysts, are introduced.
2.1 Introduction

In Chapter 1, systems capable of inducing DNA strand breaks were introduced, including the natural metallo glycopeptide bleomycin. In the presence of both dioxygen and iron, both single strand and double strand cleavage of DNA is observed, the latter being responsible for the observed cytotoxicity. Single strand DNA cleavage is the process, in which one scission is introduced in one of the two DNA strands in one cleavage event. Double strand DNA cleavage denotes the process, in which two scissions are introduced to each of the strands of the DNA double helix in a single cleavage event. Both processes are represented in Scheme 2.1.

Scheme 2.1 Representation of the two different cleavage pathways. Clockwise; single strand cleavage (ssc) pathway and counter clockwise; direct double strand cleavage (dsc) pathway. Note that both pathways will result in the formation of the same product ultimately; a DNA fragment with cuts in opposite strands.

With a single strand cleavage pathway, the DNA fragment undergoes a cleavage event, which results in a cut in one of the two DNA strands. Based on statistics, prolonged single strand cleavage results in a second single strand cleavage event taking place opposite to a strand break introduced earlier. This results in DNA fragmentation due to the presence of two cuts opposite to each other in both strands (Scheme 2.1, ssc pathway). In a double strand cleavage pathway, both of the DNA strands are cut at the same moment or in rapid succession (Scheme 2.1, dsc pathway). Although both pathways introduce double strand breaks ultimately, it is important to note that these are two inherently different processes.

The pathway that is responsible for the induction of strand breaks is of more interest than the actual degeneration of DNA, as the direct double strand cleavage pathway is presumably responsible for the cytotoxicity of bleomycin (see Chapter 1, Section 1.3). To discriminate between a single strand cleavage pathway and a double strand cleavage pathway, an assay is used, which employs a special type of DNA (a plasmid DNA). This plasmid DNA allows a qualitative and a quantitative measurement of both processes.

In the first part of this chapter, plasmid DNA and its features are introduced. How this plasmid DNA is used to discriminate between both aforementioned processes will be discussed. A statistical analysis of the results is required to quantify the number of DNA strand breaks, both single and double stranded. Since this is not trivial, this matter will be discussed in detail in the second part of this chapter. The final part of this chapter is dedicated to introduce new methods, which can be used for analyzing the DNA cleaving properties of (new) catalytic systems.
2.2 Use of plasmid DNA as benchmark substrate

The first reports of DNA cleavage using bleomycin both in vivo\cite{10} and in vitro\cite{11,12} were published in 1969. A fragment of linear DNA was used as a substrate in the oxidation reaction. An assay, which relied upon velocity sedimentation with sucrose gradients, was used to analyze the DNA products. In this assay, the sedimentation is a function of the number of strand cuts in the DNA. However, a major drawback is that a substantial number of cleavage events, both single and double stranded, are required for analysis. In practice, this means that prolonged reaction times or high concentrations of bleomycin are required. Moreover, it is difficult to distinguish whether the observed double strand cuts in the DNA are generated directly or are the result of two independent single strand cleavage events.

A significant advance was the use of covalently closed circular DNA (or plasmid DNA) instead of (a fragment of) linear DNA. Plasmid DNA (also referred to as vector when used in genetic engineering) is double stranded and is an accessory genetic element which is capable of autonomous replication independently in the cytoplasm of bacteria. Typically, the quaternary structure of plasmids is superhelical, which means that the double stranded DNA forms a compact folded structure (Figure 2.1, left). When one of the DNA strands is broken, a swivel, i.e. a free rotation site in the complementary strand opposite to the break, relieves the strain in the twisted molecule.\cite{14,15} This causes the supercoiled structure to relax into an uncoiled form, which is referred to as nicked DNA (Figure 2.1, center). When both strands of the DNA are cleaved opposite to each other, linear DNA is observed (Figure 2.1, right). Supercoiled DNA is often referred to as form I, nicked DNA as form II and linear DNA as form III. Strictly, this is not correct, as the term ‘form’ might suggest that supercoiled, nicked and linear plasmid DNA are conformational isomers of the same molecule. The nicked and linear DNA can only be accessed via (chemical) manipulation of the initial supercoiled DNA. Therefore, supercoiled, nicked and linear DNA are different types of plasmid DNA, rather than different forms of plasmid DNA.

![Figure 2.1](image-url) Electron microscope images of polyoma plasmid DNA. Supercoiled DNA (left), nicked DNA (center) and linear DNA (right). Adapted with permission from 15. Copyright Vinogard and Lebowitz, 1966.Originally published in The Journal of General Physiology 49: 103–125, 1966.

The sedimentation rates of supercoiled and nicked DNA differ significantly, which allowing a faster screening of bleomycin in inducing strand breaks in DNA.\cite{16} However, sedimentation analysis remains a time consuming process, limiting its application. In contrast, gel electrophoresis allows for the separation of supercoiled, nicked and linear DNA in one procedure. Furthermore, this size exclusion technique is faster, more reliable, and experimentally simpler than velocity sedimentation. The supercoiled, nicked and linear DNA can be separated from each other, based mainly on their relative difference in size,\cite{17,18} although the relative migration rates of the three types are also a function of the applied voltage, buffer concentration, agarose concentration and the amount of super helical twists.
in the DNA. For most plasmids, the order of migration is supercoiled >> linear > nicked, represented schematically in Figure 2.2a. The use of this technique allows a first qualitative indication of the cleavage pathway followed (Scheme 2.1); all three types of DNA are visible at an agarose slab at the same time whenever direct double strand cuts are introduced in the DNA (e.g. with Fe(BLM)).

![Diagram of DNA migration](image)

**Figure 2.2** a) Supercoiled, nicked and linear DNA can be separated with gel electrophoresis based on their relative size. The schematic representations of the three types of DNA are depicted on the right hand side. b) Structure of ethidium bromide (EtBr).

After the electrophoresis, the gel is stained with ethidium bromide (EtBr, Figure 2.2b) to visualize the DNA bands in the gel. Ethidium bromide intercalates into the DNA and then has an increased fluorescent quantum yield compared to free ethidium bromide in solution. By irradiation at 302 nm, the now fluorescent bands of DNA can be made visible and photographed. By comparing the intensities of the individual bands, the relative amounts of supercoiled, nicked and linear plasmid DNA can be determined.

It should be noted that the EtBr uptake by supercoiled DNA compared to nicked and linear DNA is restricted. Already in 1968, Bauer and Vinograd reported about the interaction of EtBr with closed circular SV40 DNA. They found that there is dependence on the concentration of EtBr and the affinity of supercoiled DNA for this fluorescent dye. At low concentrations of EtBr, the affinity of EtBr for supercoiled DNA is higher than for nicked DNA (and linear DNA). EtBr intercalates into the supercoiled DNA and unwinds the right-handed superhelix. The degree of unwinding influences the fluorescence response, and thus the concentration of EtBr determines the observed fluorescence. However, both nicked and linear DNA do not display superhelicity and this process does not occur with these types of DNA. In contrast, at high EtBr concentrations, the EtBr forms left handed superhelices in the supercoiled DNA. Moreover, EtBr is bound externally to the DNA as well. The compact folded supercoiled DNA has less external surface than the open accessible nicked and linear DNA. As a consequence, less EtBr is bound externally to supercoiled DNA than to nicked or linear DNA and hence a lower fluorescent response is observed. In other words, at high concentrations of EtBr, the fluorescence of the supercoiled DNA is directly proportional to the amount of material, when compared to nicked and linear DNA, albeit systematically lower than for the latter two types. The superhelicity unwinding aspect of supercoiled DNA does not play a role at high concentrations of EtBr in the fluorescence response, whereas at low concentrations it has a significant influence on this parameter.

A correction factor is needed to compensate for this reduced EtBr uptake capacity of supercoiled DNA. A list of known correction factors of different plasmids is given in Table 2.1. Unfortunately, in the literature on DNA cleavage it is more the rule than the exception that wrong or no correction factors have been employed. Most striking and fundamentally wrong is the use of correction factors, that are an average of the correction factors available in the literature. It must be stressed that a correction factor is an intrinsic property of the
individual plasmid DNA (demonstrated in Table 2.1) and can, therefore, never be used for other plasmid types.

**Table 2.1** Correction factors for the reduced EtBr uptake capacity of supercoiled plasmid DNA.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Correction factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM2</td>
<td>1.42 ± 0.09</td>
<td>2, 29</td>
</tr>
<tr>
<td>ΦX174</td>
<td>1.47 ± 0.08</td>
<td>30</td>
</tr>
<tr>
<td>pBR322</td>
<td>1.22 ± 0.04</td>
<td>31, 32</td>
</tr>
<tr>
<td>SV40</td>
<td>1.04 ± 0.04</td>
<td>32</td>
</tr>
<tr>
<td>pUC18</td>
<td>1.31 ± 0.04</td>
<td>This Chapter</td>
</tr>
</tbody>
</table>

*a A value of 1.05 ± 0.02 also has been reported.*

Table 2.1 displays the correction factors of plasmids, which are suitable and have been used for studying DNA cleavage. However, a major drawback of many of these plasmids is that they are low copy number plasmids, *i.e.* a low number of copies are maintained in bacterial cells, limiting the available amount of DNA.13 Plasmids have been modified extensively to increase the copy number. From a practical point of view the advantage of this is that large quantities of DNA can be isolated. This is convenient for activity studies, as considerable amounts of DNA are consumed rapidly. Therefore, in our studies, the high copy plasmid pUC18 was used.33-35 Since no correction factor had been determined for this particular plasmid, this property was determined independently.

The pUC18 plasmid was linearized with a restriction enzyme (PstI), which has only one unique restriction site in this plasmid. To an identical sample of pUC18 an equivalent amount of water was added, thus leaving the supercoiled DNA intact. After incubation for 2 h and subsequent heat inactivation for 20 min, both samples were loaded on an agarose gel and after electrophoresis the gel was stained in 1.0 μg/mL EtBr bath (*vide supra*). A clear difference in fluorescence intensity was observed between (undigested) supercoiled DNA and (digested) linear DNA (Figure 2.3). From this difference a correction factor of 1.31 ± 0.04 was determined based on the average of 30 identical samples.

![Figure 2.3 Agarose gel slab after gel electrophoresis and staining with EtBr; Odd lanes: supercoiled pUC18 plasmid DNA, no restriction enzyme added; even lanes: linearized DNA prepared from restriction digest of supercoiled pUC18 plasmid DNA with PstI. From the difference in intensity the correction factor for the reduced uptake capacity of EtBr could be determined.](image)

### 2.3 Statistical analysis

Plasmid DNA allows for a facile comparison of the two possible DNA cleaving pathways, *i.e.* a single strand cleavage pathway and a double strand cleavage pathway. If a cleaving agent generates only single strand cuts, the first cut event will generate nicked DNA from
supercoiled DNA. To form linear DNA from nicked DNA, a second oxidation event has to occur on the second strand, opposite to the first break. The two DNA strands do not have to be cleaved directly opposite to each other. When two individual cuts in the DNA are within a number of nucleotides, the forces stabilizing the double helical structure may be sufficiently weak that these two strand breaks cause a linearization of the DNA. Previous studies established that two independent cuts should be in opposite strands within 2\textsuperscript{36,37} or 3\textsuperscript{38} base pairs. However, some invalid assumptions were used, making these results rather doubtful. Later, Freifelder and Trumbo determined with more careful experiments that the critical point lies at 16 base pairs.\textsuperscript{39} As a consequence, two strand breaks, in opposite strands, within this 16 base pair region, destabilize the double stranded helical structure sufficiently, resulting in the formation of linear DNA.

In practice, this means that the first break in the plasmid DNA, induced by a typical single strand cleaving agent, forms nicked from supercoiled DNA. A second break does not immediately result in the formation of linear DNA from nicked DNA. Rather, multiple single strand cleavage events will eventually give rise to double strand cleavage of the plasmid DNA molecule. The number of double strand DNA cuts which is to be expected via this pathway can be calculated using statistics. When the cleavage process is selective and/or the cleaving agent displays double strand cleaving properties (e.g. Fe(BLM)), more linear DNA is produced than can be accounted for on a statistical basis.

As mentioned before, both single strand and double strand cleavage processes produce linear DNA. To differentiate between both processes and in order to determine whether the linear DNA was produced via single strand cleavage pathway or a double strand cleavage pathway, a statistical analysis of the results can be used. The average number of single and double strand cuts in a DNA molecule can be calculated with a Poisson distribution.\textsuperscript{40} The general Poisson formula is displayed in Equation 2.1:

\[
f(k) = \frac{\mu^k e^{-\mu}}{k!}
\]

\textbf{Equation 2.1} General Poisson formula, where \( \mu \) is the number of events which can be expected on average, when \( f(k) \) is the probability that \( k \) events are measured.

In the case of a double strand cut in DNA the number of events is 1 (\( k = 1 \), one double strand break in the DNA) and \( \mu \) is the average number of double strand cuts in the DNA \((m)\). So the formula can be rewritten to Equation 2.2.

\[
f_{III} = m \times e^{-m}
\]

\textbf{Equation 2.2} Average number of double strand cuts in one DNA molecule, calculated from the fraction of linear DNA \((f_{III})\). This fraction of linear DNA can be determined by gel electrophoresis.

It should be noted that this formula has a maximum at \( f \approx 0.37 \), which becomes clear when the Poisson distribution for \( k = 1 \) is plotted (Figure 2.4, solid line). The maximum of this distribution lies at 0.37 for \( k = 1 \). This means in practice that at any given time only up to 37% of DNA can be present with only one double strand cut.

The Poisson distribution for DNA with two double strand cuts can be written as:

\[
f_{III*} = 0.5 \times m^2 \times e^{-m}
\]

\textbf{Equation 2.3} Distribution for DNA with two double strand cuts, where \( f_{III*} \) is the fraction of the DNA with double strand cuts.
Figure 2.4 Graphical representations of the Poisson distribution for $k = 1$ (solid line) and $k = 2$ (dashed line). This corresponds to the distribution of DNA molecules with one (solid line, Equation 2.2) and two (dashed line, Equation 2.3) double strand cuts.

This means in practice that when 37% of the DNA molecules have one double strand cut, around 18% of the DNA molecules have two double strand cuts (Figure 2.4, dashed line). When plasmid DNA has two or more double strand cuts, it is fragmented into small pieces, without a distinct size, assuming a non-sequence selective cleavage process. These DNA fragments will appear as a smear on the agarose gel, precluding accurate quantification. We have chosen to use 30% of linear DNA as a maximum for analysis, as the amount of DNA with 2 or more cuts remains within an acceptable range (i.e. < 8%, Figure 2.4).

The total number of cuts, both single strand and double strand, can be calculated from the remaining fraction of supercoiled DNA. Thus, the number of events $k$ in Equation 2.1 corresponds to 0 and the total number of cuts consists of $m$ (number of double strand cuts) and $n$ (number of single strand cuts) (Equation 2.4).

\[ f_I = e^{-m-n} \]  

Equation 2.4 The total number of strand cuts (both single ($n$) and double ($m$)) in one DNA molecule calculated from the remaining fraction of DNA without any cuts. This corresponds to the remaining fraction of supercoiled DNA ($f_I$).

By combining Equation 2.2 and Equation 2.4 the average amount of single and double strand cuts per DNA molecule can be calculated. The ratio between the average amount of double strand cuts ($m$) and single strand cut ($n$) is the key to differentiating between a single or a double strand cleavage pathway. In 1969, Freifelder and Trumbo derived Equation 2.5, which describes the average amount of single strand cuts, which are required to yield a double strand cut in one molecule of DNA. This is also known as the Freifelder-Trumbo relationship.

\[ m = \frac{n^2(2h+1)}{4L} \]  

Equation 2.5 The Freifelder-Trumbo relationship, with $h$ as the maximum distance in base pairs between nicks on opposite strands to generate a double strand cut (i.e. 16) and $L$ the total number of base pairs of the DNA used (2686 base pairs for pUC18 plasmid DNA).

It can be calculated from this Freifelder-Trumbo relationship that initially one out of every 100 cuts in pUC18 plasmid DNA is a double strand cut. Indeed, by using these statistical
formulas, Povirk and co-workers have demonstrated that bleomycin induces direct double strand cuts.\textsuperscript{40} They have calculated the average single and double strand cuts in Col E1 plasmid DNA (6646 base pairs) induced by bleomycin, by using Equation 2.2 and Equation 2.4. When the amount of double strand cuts $m$ is plotted as a function of the amount of single strand cuts $n$, a linear correlation is observed (Figure 2.5). The dashed line represents the Freifelder-Trumbo relation (Equation 2.5). It becomes evident from this data that the double strand breaks induced by bleomycin are not the result of two independent sequential single strand breaks, but rather the result of independent direct double strand breaks. Treatment of DNA with $^{60}$Co $\gamma$-rays resulted in less double strand cuts (Figure 2.5). It is known that $\gamma$-irradiation of DNA results in both single and double strand cuts,\textsuperscript{42,43} but it is apparent from these data that bleomycin is much more effective in introducing double strand cuts in DNA. So in order to determine whether a DNA cleavage agent is capable of inducing direct double strand breaks, the data obtained should be compared with data obtained from the Freifelder-Trumbo relationship.

![Figure 2.5 Production of single- and double strand breaks in Col E1 DNA by bleomycin, as determined from conversion of supercoiled DNA into linear and nicked DNA. Dashed line is the Freifelder-Trumbo relation. Adapted with permission from 40; copyright 1977, Oxford University Press: Nucleic Acids Research.](image)

When a DNA cleaving agent does not induce direct double strand cuts, but only displays single strand cleavage, Equation 2.4 simplifies to Equation 2.6, provided that no linear DNA is present yet.

\begin{equation}
  f_I = e^{-n}
\end{equation}

\textbf{Equation 2.6} Average number of single strand cuts ($n$) in a DNA molecule, calculated from the fraction of supercoiled DNA ($f_I$).

After extended single strand cleavage, linear DNA will be formed as well. This linear DNA is the results of two independent single strand cuts in the DNA within a 16 base pair region. Equation 2.7 takes into account this Freifelder-Trumbo relationship.\textsuperscript{31}
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\[ f_I + f_{II} = \left[1 - n(2h + 1)/2L\right]^{1/2} \]

**Equation 2.7** Average number of single strand cuts \((n)\) in a DNA molecule, calculated from the fractions supercoiled \((f_I)\) and nicked \((f_{II})\) DNA. This equation takes the maximum distance in base pairs between nicks on opposite strands to generate a double strand cut \(i.e. 16\) base pairs \((h)\) and the total number of base pairs of the DNA used \((L)\) into account.

Using this average number of strand cuts in a DNA molecule instead of only reporting the relative quantities of each type of DNA (supercoiled, nicked and linear DNA) allows a more simple comparison between e.g. different DNA cleavage conditions. Hertzberg and Dervan used this approach in their study of Fe\(^{II}\)(MPE).\(^{31,44}\) They used a wide variety of reactive oxygen scavengers (such as SOD and catalase, see Chapter 3) to gain insight into the nature of the reactive oxygen species. The effect of a scavenger was reflected immediately in the average number of strand cuts in a DNA molecule. Using this method, they found an involvement of both superoxide and hydrogen peroxide in the catalytic cycle.

### 2.4 Other methodologies

When supercoiled DNA is used as a benchmark substrate, a single strand cut will result in an immediate formation of nicked DNA. Linear DNA will be formed eventually after prolonged cleavage. A direct double strand cut in the supercoiled DNA will result in direct formation of this linear DNA. However, in principle, linear DNA can be formed via other direct double strand pathways also, namely via the direct double strand cleavage of nicked DNA and linear DNA (Scheme 2.2). A direct double strand cut in nicked DNA will result in formation of linear DNA, whereas the same process will result in fragmentation of the DNA substrate when it takes place on linear DNA. In order to examine these possible pathways fully, new methods are required.

![Scheme 2.2](image) Single strand cleavage (ssc) pathway and double strand cleavage pathway (dsc) for the various types of plasmid DNA.

**2.4.1 Nicked DNA as benchmark substrate**

Isolated nicked DNA is needed to investigate the possibility for nicked DNA as a substrate for direct double strand cleavage. The rate of formation of linear DNA is a measure for the amount of double strand cleavage events. Earlier studies focused on the generation of nicked DNA by treatment of supercoiled DNA with pancreatic DNAsuc\(^{14,15}\) short wavelength UV light\(^2\) or X-ray irradiation.\(^38\) However, these procedures generate ill-defined numbers of random single strand breaks in the DNA and formation of linear DNA could not be excluded. Currently, restriction enzymes, which are capable of hydrolyzing only one of the two strands of DNA and thus the formation of only nicked DNA without formation of linear DNA, are available commercially. This approach enables isolation and use of
nicked DNA with a specific number of nicks, depending on the number of restriction sites in the DNA.

When pUC18 was treated with commercially available restriction enzyme Nt.BstNBI, the plasmid was digested to nicked DNA. Since pUC18 has four restriction sites for this enzyme, the nicked DNA has four single strand cuts. DNA digested on large scale could be isolated using standard DNA isolation and purification techniques. The purity of the isolated nicked DNA was confirmed by gel electrophoresis (Figure 2.6). The stability of the nicked DNA is rather low, which means that after isolation, it should be stored at -20°C and should be employed in reactions promptly.

![Figure 2.6 Isolation of nicked DNA, lane 1 untreated supercoiled pUC18 plasmid DNA; Lane 2 pUC18 plasmid DNA digested with PstI restriction enzyme (linear DNA); Lane 3 and 4 pUC18 plasmid DNA after digestion with Nt.BstNBI and isolation (nicked DNA).](image)

2.4.2 Linear DNA as benchmark substrate

Examination of possible double strand cleavage of linear DNA is somewhat more difficult. A DNA cleavage agent without DNA sequence selectivity will fragment linear DNA into smaller pieces. This causes a smear on the gel, making quantification more difficult. However, it is possible to quantify the conversion of linear DNA into smaller fragments of DNA by making use of external reference DNA. The decrease in fluorescence of the band of linear pUC18 plasmid in time with respect to this external reference DNA is a measure for the number of double strand cuts.

This approach requires the use of a well-defined linear fragment of DNA as substrate. For this purpose linearized pUC18 was selected, since it is easily accessible by digestion of supercoiled pUC18 with PstI (Figure 2.6). Linear DNA was isolated on large scale by making use of standard DNA isolation and purification techniques, similar to those for nicked DNA. Another linear DNA fragment can function as the external reference, which can be added to the reaction mixture prior to gel electrophoresis. Initial attempts focused on the use of a pBAD vector (4100 base pairs). Isolation of this plasmid was found to proceed smoothly with standard DNA isolation techniques. To use this plasmid as an external reference, the linear type DNA is required, since the band belonging to the supercoiled pBAD is too close to the band belonging to the nicked pUC18, complicating analysis. Although the digestion of pBAD with PstI on large scale proceeded smoothly, the purification of the linear pBAD was found to be quite troublesome. Analysis of the material after the digestion shows a complete conversion to linear DNA, but hardly any of this linear could not be isolated. It is likely that the linear pBAD plasmid is too unstable. Therefore, another plasmid was chosen, i.e., pTWIN (7375 bp). Although this is a high copy plasmid, the isolated yields were rather disappointing initially. When the growth and isolation conditions were changed to low copy plasmid conditions more satisfactory results were
obtained. Presumably, the production of pTWIN in XL1Blue is not very efficient and another host is likely to be more suitable. The linear DNA could be isolated after digestion with PstI, albeit in low yields (< 10%).

2.5 Summary and conclusions

This chapter provides an overview of the methods which are used to investigate the DNA cleavage properties of DNA cleaving agents. The assay using plasmid DNA as a benchmark substrate is a facile method, both in terms of experimentation and analysis. The plasmid DNA is supercoiled initially. When one of both DNA strands is cut, the superhelicity of the molecule unwinds and relaxed circular DNA is obtained (nicked DNA). When another cleavage event takes place in the opposite strand within 16 base pairs of the first cut, linear DNA is formed. The amount of DNA cleavage products can be quantified by gel electrophoresis followed by staining with EtBr. Although this assay has been used for several decades now, some important aspects are often overlooked frequently, resulting in errors in analysis. Most notably is the incorrect usage of a correction faction for the reduced uptake capacity for EtBr of supercoiled DNA, which could result in errors up to 30% in quantification of the relative amounts of supercoiled, nicked and linear DNA. In this research we make use of pUC18 plasmid DNA. The correction factor for this plasmid was determined to be 1.31.

A single strand cut in supercoiled DNA results in the formation of nicked DNA, whereas a double strand cut results in linearization of the DNA. This is a first qualitative measure for a double strand cleavage pathway and a single strand cleavage pathway. To quantify the average number of single- and double strand cuts in each DNA molecule, statistical formulas, based on a Poisson distribution, are introduced. A careful statistical analysis of the cleavage results obtained (i.e. the relative amount of supercoiled, nicked and linear DNA), allows to distinct whether the linear DNA is formed via a single strand cleavage pathway or that a double strand cleavage pathway is involved also.

In principle, double strand cleavage can occur in nicked DNA and linear DNA also. To examine these competing routes, nicked and linear DNA were isolated on large scale. These can be used as substrates in cleavage reactions in place of supercoiled DNA. By examination of the product distribution, additional information can be obtained of possible strand cleavage activity of complexes under investigation.

In conclusion, this chapter provides an overview of the methods and materials used in the study of DNA cleaving systems, such as the iron N4Py complexes. In addition to supercoiled DNA, nicked or perhaps linear DNA can be used as substrates in the reaction also, which can provide additional information about the double strand cleavage activity of iron N4Py complexes.

2.6 Experimental section

General remarks

pUC18 and pTWIN plasmid DNA, isolated from E. Coli XL1 Blue, and pBAD, isolated from E. Coli TOP10, were purified using QIAGEN® maxi kits (pTWIN plasmid under low copy conditions). Concentrations were determined by both dilution gels and UV spectroscopy (A260). Restriction enzymes and restriction buffers were purchased from New England Biolabs (NEB). The activities of the restriction enzymes are denoted in units (U). Agarose used for the agarose slab gels was purchased from Invitrogen. A solution of
number IV dye, consisting of 0.08 % bromophenol blue and 40% sucrose (6 × conc.) was added to the samples prior to electrophoresis. All gels were run on 1.2 % agarose slab gels for at least 90 min at 70 V. Gels were stained in a ethidium bromide bath (1.0 μg/mL) for 45 min, followed by destaining for 1 min in gel running buffer. Pictures of the agarose slab gels were taken with an Appligene High Performance CCD camera. The bands on the film were quantified using the software program Scion Image 4.0.3.2. The contrast of the pictures of the agarose slab gels, used as illustrations in this chapter, is inverted for clarity.

Determination of the correction factor for the reduced ethidium bromide uptake of supercoiled pUC18 plasmid DNA

A stock solution (consisting of pUC18 plasmid DNA, restriction buffer 3 (NEB) and water) was divided in 60 identical samples (0.1 μg pUC18 plasmid DNA per sample). PstI restriction enzyme (6 U; 60 fold excess) or an identical volume of water was added to the samples to a final volume of 50 μL. The samples were incubated for 2 h at 37°C, followed by heating at 80°C for 20 min. After cooling to room temperature, 10 μL of loading buffer was added. 15 μL of each sample was loaded onto an agarose gel. After running and subsequent staining of the gel in an EtBr bath, the intensities of the bands of both supercoiled and linear DNA were compared. From the relative difference in intensity between these bands, a correction factor of 1.31 (± 0.04) was determined.

Isolation of nicked pUC18 plasmid DNA

To a solution of supercoiled pUC18 plasmid DNA (206 μg) in water and restriction buffer 3 (NEB) was added Nt.BstNBI (250 U; 1.2 fold excess), to a total volume of 500 μL. The mixture was incubated at 55°C for 4 h, followed by heating to 80°C for 20 min. After cooling to room temperature, the mixture was added to a premixed solution of buffers 1, 2 and 3 of a QIAGEN® maxi-kit (10 mL each, SDS removed via centrifugation). The mixture was loaded on a column (QIAGEN® maxi kit) and the manufacturer’s protocol was followed. Yield 61 μg (30%) of DNA after isolation. The purity of the nicked pUC18 plasmid DNA was confirmed by gel electrophoresis (Figure 2.6).

Isolation of linear pUC18 plasmid DNA

The same procedure as was described for the isolation of nicked pUC18 plasmid DNA was followed, however the supercoiled DNA was digested with 240 U (1.2 fold excess) of PstI restriction enzyme. The reaction mixture was incubated at 37°C for 4 h. After isolation, 60 μg (29 %) of linear pUC18 DNA was obtained.

Isolation of linear pTWIN plasmid DNA

The procedure as described for the isolation of nicked pUC18 plasmid DNA was used, except starting with 175 μg of supercoiled pTWIN DNA and addition of 400 U (2.3 fold excess) of PstI restriction enzyme to a total volume of 1 mL. The mixture was incubated at 37°C for 4.5 h. Yield 11.2 μg (6.4 %) of linear pTWIN DNA.

2.7 References and notes

1 See Appendix for abbreviations used.
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39 D. Freifelder, B. Trumbo, Biopolymers 1969, 7, 681-693.
According to the manufacturer’s protocol the use of Nt.BstNBI restriction enzyme requires 10 times excess for a full restriction within 1 h. However, a longer reaction time with lower enzyme amounts results also in full restriction.