Eur Biophys J (1998) 27: 147–151

ARTICLE

Maurice Durand · Claire Gondeau
Jean Claude Maurizot

Interaction of methyl green with an oligonucleotide in intramolecular duplex and triplex conformations

Circular dichroism studies

Received: 15 July 1997 / Accepted: 22 September 1997

Abstract Interaction of methyl green with the oligonucleotide 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC (where [T_4] is a nucleotide sequence of four thymines) in hairpin duplex and in intramolecular triplex structures has been studied by circular dichroism. We found that methyl green binding to the duplex form shows a complex pattern, exhibiting an exciton contribution when the number of bound molecules increases. Differences between this pattern and previously published results on other DNAs reveals the presence of different types of complexes. In contrast to previous findings with the triple helix poly(dA).2poly(dT) we show that the methyl green is not totally excluded from this triplex structure made of Pur:Pur:Pyr triplets.

Key words Methyl green · DNA triplex · Pur:Pur:Pyr triplet · Circular dichroism

Introduction

Methyl green (MG) belongs to the class of triphenylmethane dyes. MG has long been used as a DNA stain in histochemistry because it is stabilized by complexation with B-DNA, while the free dye in solution spontaneously rearranges into the colorless benzenoid carbinol. There has been debate about the binding mode of MG. It has been suggested that binding occurs by intercalation and that binding occurs across the minor groove and/or the major groove of DNA. Previous studies have shown that this dye possesses a greater affinity for AT-rich DNA than for GC-rich DNA (Krey and Hahn 1975; Müller and Gautier 1975). It was also shown that binding of MG to DNA is predominately related to ionic contacts while some non-ionic forces were believed to be of minor importance (Krey 1980). More recently, using linear and circular dichroism methods, an interesting approach was proposed to investigate how the MG interacts with B-DNA (Kim and Nordèn 1993). Taking advantage of the fact that MG is excluded from binding to triplex poly(dA).2poly(dT), where the third strand fills the major groove of the double helix, the authors present evidence for a major groove binding geometry of MG bound to the poly(dA).poly(dT) duplex. The chemical structure of MG, which is formed by 3 phenyl rings arranged in a propeller-like conformation, is shown in Fig. 1. Though non-planar owing to steric hindrance (Gust and Mislow 1973) MG can be considered as having a pseudo-C_{2v} symmetry. Consequently the free molecule has no circular dichroism (CD) spectrum. However, a CD spectrum was detected when the dye is bound to DNA (Nordèn and Tjerneld 1977). We use this property with the oligonucleotide 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC (where [T_4] is a nucleotide sequence of four thymines), which can adopt (Fig. 1) a hairpin duplex structure with a dangling single-stranded tail or an intramolecular triplex structure in solution (Durand et al. 1997, manuscript submitted for publication), to show by circular dichroism spectroscopy that the binding of MG to DNA duplex exhibits several types of complexes with duplexes and that binding to the triplex is not excluded. The characteristic CD spectra of the MG-oligonucleotide complexes are shown.

Based on work presented at the 2nd European Biophysics Congress, Orléans, France, July 1997

M. Durand · C. Gondeau · J. C. Maurizot (✉)
Centre de Biophysique Moléculaire, Université Orléans,
Rue Charles Sadron, F-45071 Orléans Cedex 2, France

Materials and methods

Solution preparation

The 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC or 5′-dGGAAAAGG-[T_4] oligonucleotides purified by ion exchange HPLC were purchased from Oncor-Appligene. Oligonucleotide solutions were prepared with a buffer containing 10 mM sodium cacodylate and 0.2 dis-

odium EDTA, pH 7.0. The samples containing 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC were heated at 90°C for 10 min and allowed to come to room temperature slowly to overcome formation of competing secondary structures. The various concentrations of $MnCl_2$ salt in sample solutions were obtained by additions of concentrated solutions of salt. The concentration of oligomers, expressed per nucleotide, were determined from high-temperature absorbance at 260 nm, i.e. with DNA in the denatured state, assuming as extinction coefficients 8500 $M^{-1}cm^{-1}$ for T and C and 15000 $M^{-1}cm^{-1}$ for A and G.

MG was obtained from Sigma. Because the conversion of MG into the benzenoid carbinol is favored by pH greater than 5, working solutions were prepared daily by dissolving dye in a buffer at pH 5. The concentration was determined spectrometrically using the molar extinction coefficient ε_{638} = 78,500 M^{-1} cm^{-1}.

Circular dichroism spectroscopy

Circular dichroism measurements (CD) were carried out on a Jobin-Yvon Mark IV dichrograph. Data acquisition and analysis were performed on a computer interfaced to the spectrometer. Optical cells with a pathlength of 1.0 cm were used. Spectral titrations were carried out at 3°C. The temperature of the cell was adjusted with a circulating refrigerated water bath and held constant to ± 0.5°C. During the experiments nitrogen was continuously circulated through the cuvette compartment. Each CD spectrum was an average of two scans with the buffer blank, which was also an average of two scans, subtracted, and we checked for possible base line shifts. The concentration used to calculate the CD amplitude was that of the nucleotide unit.

Results and discussion

In previous studies (Durand et al. 1997, manuscript submitted for publication), we investigated, at 3°C, the effect of the cations Mg^{2+} and Mn^{2+} on the 32-mer oligonucleotide 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC. An NMR study has revealed that in the presence of 5 mM Mg^{2+} this oligomer folds back on itself twice and forms (at pH 7) a stable pur.pur:pyr triplex (Fig. 1). The first eight residues fold back to form an antiparallel Watson-Crick hairpin duplex with a [T_4] loop and the 3′-terminal eight residues, which are connected to the Watson-Crick hairpin duplex by a second [T_4] loop, form Hoogsteen hydrogen bonding in the major groove of the Watson-Crick underlying duplex. The design of the 32-mer oligonucleotide ensures that the orientation of the third purine strand is antiparallel to the purine of the duplex. We characterized the triplex state by its CD spectrum. On the basis of CD spectra we found that very small concentrations of Mn^{2+} induce changes similar to those produced by Mg^{2+}, 4 mM Mn^{2+} being enough to form the hairpin pur.pur:pyr triplex.

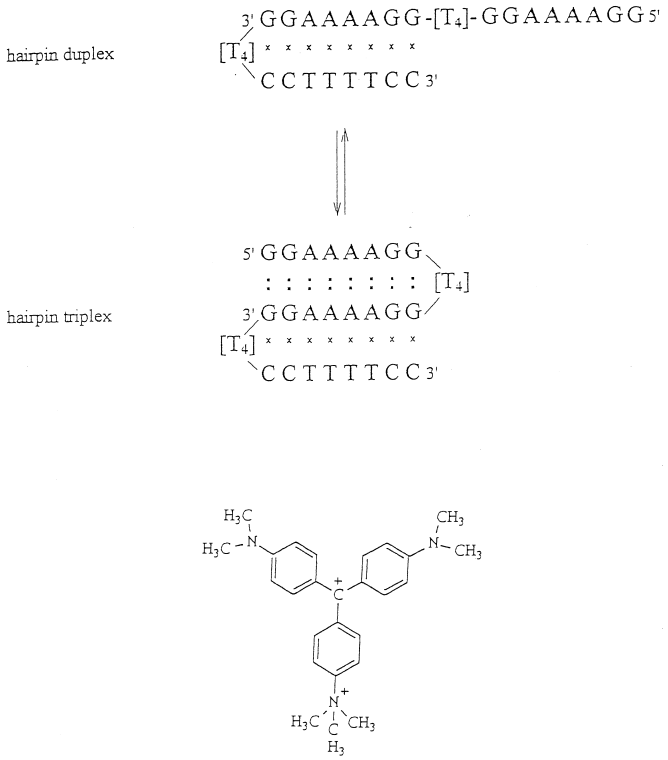

Fig. 1 Schematic representation of monomolecular structures adopted by the 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC oligonucleotide: a hairpin duplex with dangling single-stranded tail and an intramolecular triplex. Sequence of 4 thymines symbolized by -[T_4]- represents a loop region. Molecular representation of methyl green (MG)

Interaction of MG with the oligonucleotide in duplex conformation

MG absorbs prominently in the visible region with an absorption maximum at 632 nm and presents less intense absorption maxima at 421 nm, 312 nm and at 260 nm. Figure 2 shows the CD spectra obtained upon addition of MG to 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC at 3°C and in a buffer containing 10 mM sodium cacodylate and 0.2 mM disodium EDTA, pH 7.0. In this buffer and in the absence of ligand the oligonucleotide forms a very stable hairpin duplex with a dangling 5′-dGGAAAAGG-[T_4] extremity, since its denaturation occurs at 55°C (data not shown). In the 350–750 nm region where only the ligand absorbs its binding induces a CD signal. As seen in Fig. 2a, we observe the appearance of a negative CD band centered at 430 nm which increases regularly up to a saturation value close to a [MG]/[oligo] ratio equal to 12, the highest ratio used in these experiments. For this [MG]/[oligo] ratio it is clear from the shape of the binding curve (insert of Fig. 2a) and from the previously published value of the binding constant (of the order of 10^5 M^{-1}, Nordén et al. 1978) that the number of bound MG molecule per oligonucleotide is far smaller than the ratio [MG]/[oligo]. In the 550–700 nm region the induced CD

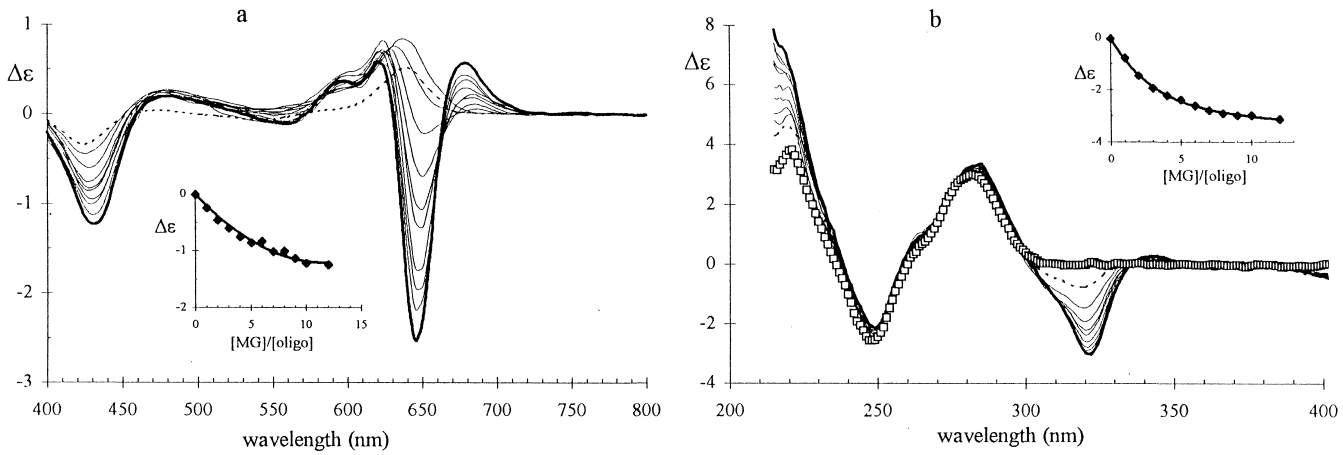

Fig. 2a,b CD spectra of solutions of 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC oligonucleotide in hairpin duplex structure, containing different concentrations of MG. **a** Spectra measured in the visible wavelength region. The r = [MG]/[oligo] ratio varying from 0.5 (- - -) to 12 (*bold solid line*). The insert of the figure, represents the spectral titration measured at 430 nm of oligonucleotide with MG. **b** Spectra measured in the UV wavelength region. The r = [MG]/[oligo] ratio varying from 0 (□□□) to 12 (*bold solid line*). The insert of the figure represents the spectral titration measured at 320 nm of oligonucleotide with MG. Measurements were made at 3°C

spectrum exhibits more complex behavior with dramatic changes in the shape of the spectrum when the ligand concentration increases. In a first step, a positive CD band centered at 640 nm increases, then decreases and shifts to 633 nm for [MG]/[oligo] = 3. At higher ratios a new negative band centered at 645 nm appears. The complex shape of the CD spectrum at high [MG]/[oligo] ratio certainly reflects the presence of an exciton contribution due to MG:MG interactions. Such exciton interactions have previously been mentioned (Nordèn et al. 1978), but the shape of the spectrum in our experiment cannot be considered as a pure exciton since it is more complex (a large negative band between two smaller positive bands). We performed a control experiment with the oligonucleotide 5′-dGGAAAAGG-[T_4] which represents the single strand 5′ extremity of the 5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC hairpin duplex. We did not observe any induced CD and we noted no change in the spectrum of 5′-dGGAAAAGG-[T_4] (data not shown). This finding shows clearly that either there is no interaction of the MG with the single strand DNA or there is an interaction which induced no CD perturbation. In any case, this demonstrates that the CD changes observed in our experiments reflect only interactions between MG and the duplex part of the dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC oligonucleotide. In the 215–350 nm region, we note the appearance of an induced band located at 320 nm, an increase of the intensity of the signal at 220 nm and a very weak increase of the band centered at 250 nm (Fig. 2b). In the oligonucleotide wavelength region during the titration we do not observe any change in the features of the CD spectra. It has been shown by circular and linear dichroism studies that MG exhibits two complexes with calf thymus DNA (Nordèn and Tjerneld 1977; Nordén et al. 1978), and similar results were observed with the synthetic polynucleotide poly(dA).poly(dT) (Kim and Nordén 1993). In studies with calf thymus DNA, the CD spectrum corresponding to the complex obtained at low [MG]/[DNA] is characterized by a positive CD band located between 550 and 700 nm and centered at around 640 nm with a shoulder at lower wavelength, while the CD spectrum obtained at higher [MG]/[DNA] ratio is defined by an almost conservative spectrum with a negative band centered at 630 nm and a positive band centered at 680 nm. Furthermore, this latter spectrum has the same shape as the CD spectrum of the MG-poly(dA).poly(dT) complex (Kim and Nordén 1993). It is noteworthy that the CD spectra of MG-DNA and MG-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC have common features at low [MG]/[oligo] ratios. In contrast, at higher ratios the CD spectrum of the second MG-DNA or the MG-poly(dA).poly(dT) complexes are remarkably different from the CD spectrum observed with the MG-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC complex. Indeed, instead of a negative band centered at 630 nm and a positive band centered at 680 nm, the CD spectrum obtained with dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC has a negative band centered at 645 nm, edged with two weak positive bands. These results suggest that several complexes are formed between MG and the nucleic acid in the duplex structure. At low MG concentration, the similarity of the induced CD spectra obtained with DNA, poly(dA).poly(dT), and the oligonucleotide we have used, might indicate that the complexation is not sequence specific. In contrast, at higher MG concentration the circular dichroism differences observed between calf thymus DNA, poly(dA).poly(dT) and dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC suggest a sequence/base composition dependence of complex formation. In agreement with this hypothesis, a study using linear dichroism (Bailly et al. 1992) clearly indicated that the binding of the MG molecule to DNA produces positive and negative linear dichroism signals at AT and GC sites respectively.

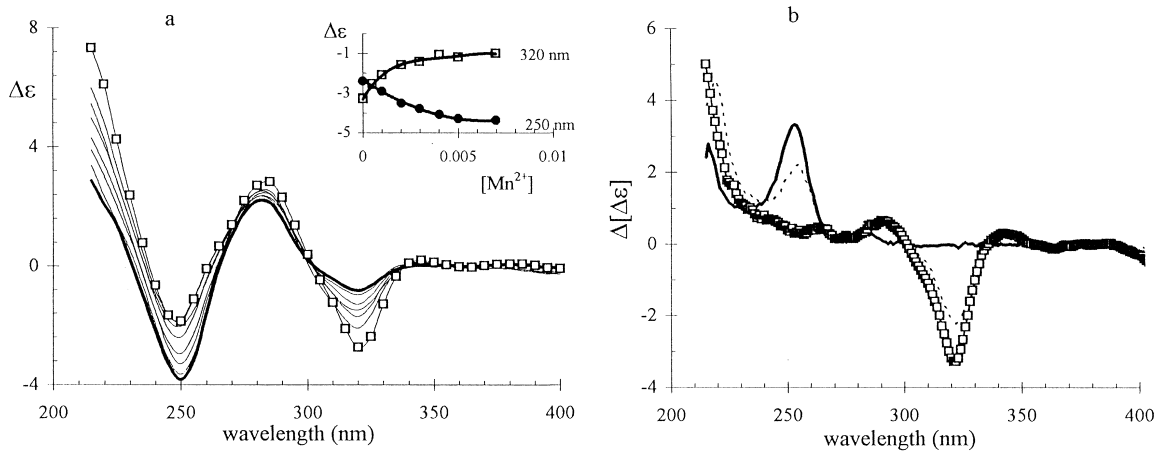

Fig. 3 **a** Effect of $MnCl_2$ on the CD spectrum of the MG-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC complex ([MG]/[oligo] = 12), in the UV wavelength region. $MnCl_2$ concentration varying from 0 (□□□) to 7 mM (*bold solid line*). The insert represents the CD signal versus Mn^{2+} concentration measured at 250 and 320 nm. **b** Difference spectra: (□□□) in the absence of $MnCl_2$ (oligonucleotide *minus* oligonucleotide in the presence of MG for a ratio [MG]/[oligo] = 12, (- - -) in the presence of 5 mM $MnCl_2$ (oligonucleotide *minus* oligonucleotide in the presence of MG at a ratio [MG]/[oligo] = 12), (*bold solid line*) in the absence of MG (oligonucleotide in the absence of $MnCl_2$ *minus* oligonucleotide in the presence of 5 mM $MnCl_2$). Measurements were made at 3°C

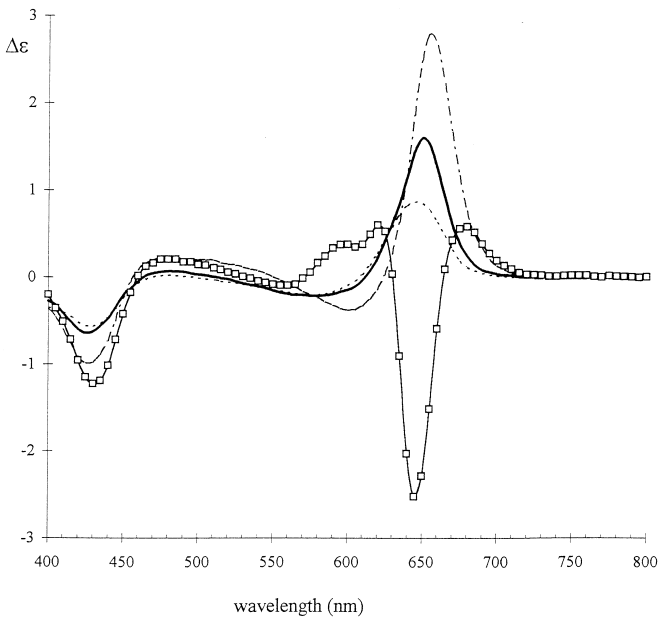

Fig. 4 In the visible wavelength region, CD spectra for the MG-5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC complex obtained for [MG]/[oligo] = 12, as a function of $MnCl_2$ concentration: (□□□) no $MnCl_2$ salt, (- · - · -) 0.5 mM, (——) 3 mM and (- - -) 7 mM

Interaction of MG with the oligonucleotide in the triplex conformation

To observe the binding of MG to the triplex form of the oligonucleotide we added increasing concentrations of $MnCl_2$ (0.5 mM to 7 mM) to the complex at a [MG]/[oligo] ratio equal to 12. Figure 3a shows the effect of the $MnCl_2$ on the CD spectrum in the DNA wavelength region. Upon addition of salt, we note an increase and a decrease of the intensity of the CD bands centered at 250 and 320 nm respectively. In the insert of Fig. 3a are shown the CD variation as a function of $MnCl_2$ concentration, recorded at 320 nm and 250 nm. Taking into account the accuracy of the experiments, we observe no significant further change beyond 5 mM $MnCl_2$. When the dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC oligonucleotide is alone, the increase in the CD band located at 250 nm is characteristic of the triplex conversion of this oligonucleotide (Durand et al. 1997, manuscript submitted for publication). In the Fig. 3b are compared the CD difference spectra (free oligonucleotide *minus* free oligonucleotide in presence of 5 mM $MnCl_2$) and (oligonucleotide complexed with MG in the absence of $MnCl_2$ *minus* oligonucleotide complexed with MG in the presence of 5 mM $MnCl_2$) at [MG]/[oligo] = 12. The positive band located between 230 and 270 nm characteristic of the triplex formation for the free oligonucleotide is also present for the bound oligonucleotide, but it is not observed in the CD difference spectrum (oligonucleotide complexed with MG *minus* free oligonucleotide) obtained in the absence of $MnCl_2$ salt. These results indicate that in the presence of MG and 5 mM $MnCl_2$, the oligonucleotide is in a triplex structure. The persistence of an induced CD signal at 320 nm is clear evidence of the formation of a complex between the oligonucleotide in the triplex structure and MG. In the visible wavelength region, the variation of the CD signal upon addition of $MnCl_2$ is more complex. In the presence of 0.5 mM $MnCl_2$, the lowest salt concentration used in this experiment, the negative CD band centered at 645 nm is completely suppressed and is replaced by an intense positive CD band centered at 655 nm and a weak negative band located around 600 nm (Fig. 4). This low $MnCl_2$ concentration is not large enough

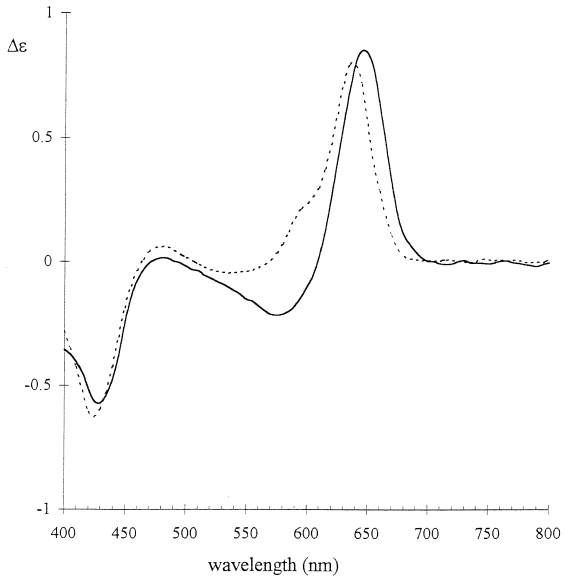

Fig. 5 Comparison of the CD spectra of the MG-5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC complex ([MG]/[oligo] = 12) in the presence (——) of 7 mM $MnCl_2$ salt (oligonucleotide in intramolecular triplex structure), and the CD spectrum of the MG-5′-dGGAAAAGG-[T_4]-GGAAAAGG-[T_4]-CCTTTTCC complex ([MG]/[oligo] = 2) in the absence (- - -) of $MnCl_2$ (oligonucleotide in hairpin duplex structure with dangling single-stranded tail)

for the formation of the triplex form. This CD change might be related to the fact that, owing to the presence of the salt, the binding density of MG on the oligonucleotide is decreased, reducing the MG:MG interaction, and consequently the exciton contribution to the spectrum. However one must notice that at this Mn^{2+} concentration the induced CD signals at 320 and 450 nm are only slightly decreased. Another hypothesis might be that the presence of Mn^{2+} leads to the disappearance of the second type of MG-oligonucleotide complex previously mentioned. At this low $MnCl_2$ concentration, the displacement of the MG by the bication Mn^{2+} might reveal the electrostatic nature of the second complex. In any case the CD change cannot be attributed to the extrusion of MG from the major groove by the third strand. We have to point out that this does not exclude such extrusion by the bication Mn^{2+}. When the salt concentration is increased, there is a decrease and a shift of the positive band towards shorter wavelengths, and the disappearance of the negative CD band as shown in the Fig. 4 for 7 mM salt concentration. At 7 mM $MnCl_2$, the highest concentration used in this experiment, it is noteworthy that the CD spectrum of the complex MG-triplex is similar to but not identical to any spectrum for MG-duplex obtained for a [MG]/[oligo] ratio smaller than two (Fig. 5). These results indicate that an MG-triplex complex exists and that in this complex the environment of the MG is not identical to that observed in the first MG-duplex complex. These experiments cannot determine whether the MG is located on AAT or on GGC or on the step between these sequences. Further work using other oligonucleotide will be necessary to answer this question. The presence of the third strand in the major groove seems to exclude this localization for the MG in the triplex form, but, of course, it does not exclude this possibility in the duplex form.

To sum up, we have presented evidence by CD, that: i) The interaction of MG with a DNA duplex shows a complex pattern and probably involves several types of complexes. ii) MG can interact with a triplex which includes AAT and GGC triads, in contrast to what has been observed for a triplex containing TAT triads. Therefore, a possible interaction of MG with the minor groove of the DNA cannot be excluded. These experiments show clearly that MG interacts with triplex DNA and that it must be used with caution as a probe of triplex formation or in DNA-protein interaction studies.

References

Bailly C, Hénichart JP, Colson P, Houssier C (1992) Drug-DNA sequence-dependent interactions analysed by electric linear dichroism. J Mol Recogn 5:155–171

Durand M, Lancelot G, Maurizot JC (1997) Spectroscopic studies of the formation of an intramolecular purine.purine:pyrimidine short triplex. (submitted for publication)

Gust D, Mislow K (1973) Analysis of isomerisation in compounds displaying restricted rotation of aryl groups. J Am Chem Soc 95: 1535–1547

Kim SK, Nordén B (1993) Methyl green, a DNA major groove binding drug. FEBS Lett 315:61–64

Krey AK, Hahn FE (1975) Studies on the methyl green-DNA complex and its dissociation by drugs. Biochemistry 14: 5061–5067

Krey AK (1980) Non-intercallative binding to DNA. Prog Molec Subcell Biol 7:43–87

Müller W, Gautier F (1975) Interactions of heteroaromatic compounds with nucleic acids. Eur J Biochem 54:385–394

Nordén B, Tjerneld F (1977) Binding of methyl green to deoxyribonucleic acid analysed by linear dichroism. Chem Phys Lett 50: 508–512

Nordén B, Tjerneld F, Palm E (1978) Linear dichroism studies of binding site structures in solution. Complexes between DNA and basic arylmethane dyes. Biophys Chem 8:1–15