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Studies on mechanism of 8-methoxypsoralen–DNA interaction in the dark

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Abstract

The interaction of 8-methoxypsoralen (8-MOP) with calf thymus DNA was studied in darkness at 25 °C and pH 7.4. The enthalpy curve for 8-MOP–DNA interaction was obtained by isothermal titration calorimetry and showed a two-step process for the interaction. According to the spectrophotometric data, it was suggested that some compaction may occur in the DNA structure at higher $[8\text{-MOP}]_t$/[DNA] ratio. Using the fluorescence quenching data, the Scatchard analysis was performed for 8-MOP–DNA interaction at the extended ranges of drug concentration. The results indicated that the first set of binding sites was occupied by 1 mol of drug bound per near eight base pairs of DNA. Also 8-MOP caused the quenching of the fluorescence emission of DNA–ethidium bromide complex. The Scatchard analysis of these data indicated the non-competitive manner for quenching. A non-displacement based quenching mechanism has been suggested for this behavior. The circular dichroism spectra also confirmed the non-intercalative binding of 8-MOP at higher concentrations accompanied by some conformational changes in DNA structure. It has been suggested that at low drug load, 8-MOP binds to DNA as an intercalator, which is an endothermic process, whereas at higher ratios of $[8\text{-MOP}]_t$/[DNA], it binds to the outside of DNA, probably in the minor groove and causes some compaction in DNA, which is the exothermic process.

Keywords: DNA; 8-MOP; Intercalation; Microcalorimetry; Binding sites

Abbreviations: Et, ethidium bromide; mM, millimolar; mMb, milli-molar base; 8-MOP, 8-methoxypsoralen or 8-methoxalen; $[8\text{-MOP}]_t$, total concentration of 8-MOP; μMbp, micro-molar base pair.

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1. Introduction

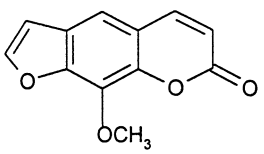
Psoralens are heterocyclic aromatic compounds derived from the linear condensation of a coumarrin nucleus with a furan ring (Foye et al., 1995). Natural furocoumarins are found in a wide variety of plants (Dollery et al., 1999) and have been used since ancient times in the treatment of various skin disorders (Guzzo et al., 1996). These compounds have shown various photobiologic activities (Parrish et al., 1974; Foye et al., 1995). They have also been used as probes of nucleic acid structure and function (Cimino et al., 1985; Ussery et al., 1992). 8-Methoxypsoralen (8-MOP), also called methoxalen, belongs to this family of compounds, which is extensively used in skin disorders (Dollery et al., 1999). The mutagenicity and genotoxicity of 8-MOP have been suggested in a variety of prokaryotic and eukaryotic cells (Clarke and Wade, 1975; Scott et al., 1976; Bridges and Mottershead, 1977; Dollery et al., 1999). It has been suggested that the biological activity of psoralens is primarily the result of the covalent binding with nucleic acids, especially DNA. A three-step mechanism for this process has been proposed (Scott et al., 1976; Isaacs et al., 1977; Dollery et al., 1999). The first step is the formation of a non-covalent molecular complex, which undergoes photobinding to DNA and then cross-linking between strands of DNA upon irradiation of the complex at 365 nm (Dall'Acqua et al., 1978). Intercalation of psoralens into the DNA helix in the dark condition has been postulated as an important intermediate of the first step (Cole, 1970; Dall'Acqua et al., 1978; Straub et al., 1981). The investigation of the intercalation geometries as well as isolation and characterization of psoralen photoadducts have been performed (Straub et al., 1981; Kanne et al., 1982; Romer and Anders, 1985). While many aspects of the interaction of these compounds with DNA under the photoirradiation have been extensively studied (Isaacs et al., 1977; Dall'Acqua et al., 1979; Cimino et al., 1985; Yeung et al., 1988; Sastry et al., 1997), there are relatively fewer reports about their interaction in darkness (Dall'Acqua et al., 1978; Hyde and Hearst, 1978; Gupta and Ali, 1984). Most of the cited works were carried out on DNA in a low concentration of 8-MOP; therefore, a study on the cited interaction in higher concentrations of 8-MOP should be examined for a better understanding of such an interaction. The involvement of more than one mechanism for binding of proflavine (Li and Crothers, 1969), some porphyrins (Fiel, 1989; Sehlstedt et al., 1994) and ethidium bromide (Waring, 1965; Pasternak et al., 1991) to DNA has been described by other investigators.

In this study, we attempt to investigate the mechanistic aspects of 8-MOP binding to DNA in darkness, especially thermodynamically and also over a broader range of ligand concentration.

2. Materials and methods

2.1. Materials

8-Methoxypsoralen has been extracted and purified from *Diplotaenia Damavandica*; a new species of the Umbelliferae, as an endemic plant to the flora of Iran by Aynehchi et al. (1999).



8-methoxypsoralen (8-MOP)

High molecular weight DNA was extracted and purified from calf thymus in our laboratory, according to the modified method of Sambrook et al. (1989) as reported previously (Bathaie et al., 1999). All other materials used were of analytical grade.

All experiments were carried out in 0.05 M Tris buffer, pH 7.4 at 25 °C, in the dark. The DNA concentrations were determined using an extinction coefficient of 6600 or 13200 M^{-1} cm^{-1} at 260 nm and expressed in terms of base molarity (Aktipis and Kindelis, 1973) or base pair molarity (McFadyen et al., 1990), respectively.

2.2. Methods

2.2.1. Isothermal titration calorimetry (ITC)

Enthalpy measurements were made at 25 °C using an LKB microcalorimeter (2277 Thermal Activity Monitor, Boromma, Sweden). The microcalorimeter was interfaced with an IBM PS/2 Model 40486 computer; thermometric DIGITAM 3 was the software program used. The enthalpy of interaction between 8-MOP and DNA was measured by transferring of 90 μl of 0.115 mM 8-MOP (in each injection) to 1.5 ml of DNA solution (0.016 milli-molar base (mMb)). The enthalpy of dilution of 8-MOP due to injection was corrected by measuring the enthalpy change after injection of 8-MOP solution into buffer solution, using identical procedures and experimental conditions. The heat released by DNA dilution was negligible.

2.2.2. Fluorimetric measurements

The fluorimetric measurements were carried out by a Shimadzu Model RF-5000 spectrofluorimeter. The instrument was operated in the energy mode. Both slit widths employed were 10 nm for the excitation and emission beams. The spectra were recorded at 60 nm min^{-1} scanning speed without filter. The emission spectrum of methoxalen was studied at $\lambda_{ex} = 345$ nm and $\lambda_{em} = 404$ nm. The fluorimetric study of the interaction of Et with DNA in the absence and presence of 8-MOP was investigated according to the method reported by Strothkamp and Strothkamp (1994).

2.2.3. Circular dichroism (CD)

CD measurements were made on a JASCO Model J-715 CD recorder at 25 °C. Data reported as molar ellipticity, $[\theta]$ (deg cm^2 $dmol^{-1}$), based on the average weight of nucleotide (AWN) which is equal to 330. The molar ellipticity was determined as $[\theta]_\lambda = (\theta \times 330)/c.l$, where c is the DNA concentration in mg/ml, l is the light path length in centimeter and θ is the measured ellipticity in degrees at a wavelength λ. The instrument was calibrated with (+)-10-comphor sulfonic acid, assuming $[\theta] = 7820$ deg cm^2 $dmol^{-1}$ and with JASCO standard nonhygroscopic ammonium (+)-10-comphor sulfonate, assuming $[\theta] = 7910$ deg cm^2 $dmol^{-1}$. The noise component in the data was smoothed using the JASCO I-715 software, including the fast Fourier-transform noise reduction method, which allows enhancement of most noisy spectra without distorting their peak shapes. The amounts of secondary structures of proteins were calculated by the J-700 for windows secondary structure estimation program (Model SSE-338).

2.2.4. Temperature scanning spectrophotometry (TSS)

DNA thermal denaturation measurements were made by means of a Gilford spectrophotometer Model 410 Digital. Temperature increasing rate, adjusted to 1 °C min^{-1} and the changes in absorption measured at 260 nm. DNA solution (0.011 milli-molar base (mMb)) was considered to be a reference while other solutions with $[8\text{-MOP}]_t/[DNA]$ molar ratio between 4 and 6 were sample solutions. The T_m calculation was performed according to the method of Li (1978) from the hyperchromicity of DNA due to the temperature.

2.2.5. Ultraviolet spectrophotometry

All other spectrophotometric measurements were made with a Shimadzu model 160-A, double-beam spectrophotometer.

3. Results

3.1. Spectrophotometry

The spectrophotometric method was used in order to determine the absorption changes of macromolecule (DNA) at 260 nm, where it acts as an acceptor for ligand (8-MOP) molecule.

Fig. 1 shows the nonlinear decrement in the absorbance of DNA at 260 nm with increasing the $[8\text{-MOP}]_t/[DNA]$ molar ratio and the curve approaches to a plateau after a ratio of ≈ 6. The minor increment also observed at 302 nm, the maximum wavelength of the methoxalen absorption, is due to its interaction with DNA (data not shown).

3.2. Quenching of the fluorescence emission of methoxalen by DNA

Fig. 2 shows the fluorescence intensity of 8-MOP in the absence and presence of DNA. The difference in the fluorescence intensity of the drug in the above two states was observed as fluorescence quenching. This means that the fluorescence emission of methoxalen decreases upon its interaction with calf thymus DNA when compared to the free state and this decrement reaches a maximum as DNA saturated with methoxalen at more than the $[8\text{-MOP}]_t/[DNA]$ molar ratio of ≈ 6. By assuming that the amount of quenching is proportional to the amount of DNA-bound 8-MOP, the difference between two curves was used to calculate binding parameters: n (the numbers of bound drug to DNA) and K (apparent binding constant).

Fig. 3 illustrates the binding isotherm curve for 8-MOP binding, as v (the extent of methoxalen moles binds to mole nucleotides of DNA) against total drug concentration, which has been derived from the fluorescence quenching data. The Scatchard plot in Fig. 3 (inset) shows a two-step curve indicating the existence of two sets of binding sites on DNA for 8-MOP. The binding parameters, n (the intercept on the abscissa) and

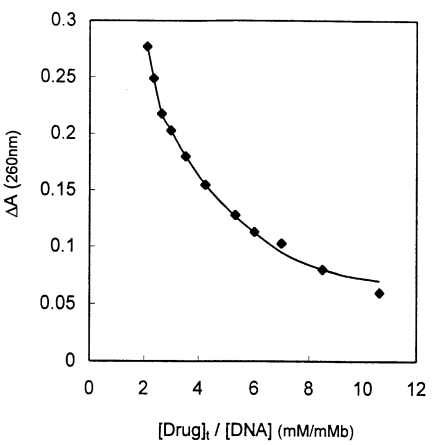


Fig. 1. The absorbance change (ΔA) of DNA at 260 nm against the molar ratio of $[8\text{-MOP}]_t/[DNA]$. Samples contained a drug with a fixed concentration (0.09 mM) and variable concentrations of DNA, ranging from 0.008 to 0.04 milli-molar base (mMb). The measurement was made in a cuvette of 1 cm path length.

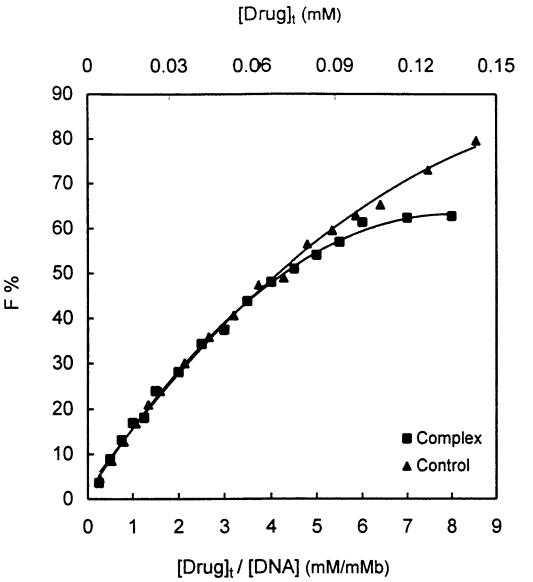


Fig. 2. Effect of DNA on the fluorescence emission of methoxalen. The concentration of DNA was held constant (0.018 mM), while that of 8-MOP varied from 0.0044 to 0.142 mM. Fluorescence emission intensity was measured at $\lambda_{ex} = 345$ nm and $\lambda_{em} = 404$ nm. Both slits were 10 nm. (▲) control (drug alone); (■) DNA–drug complex.

K (the slope of the line), for the first set of binding sites (or the intercalative sites) were determined. These values are 0.06 (i.e. 1 mol of 8-MOP binds per near eight base pairs of DNA) and 5.02×10^5 M^{-1}, respectively.

3.3. The inhibitory effect of 8-MOP on the binding of ethidium to DNA

The binding of ethidium bromide to DNA, as an intercalating dye, is associated with the enhancement in its fluorescence intensity (Waring, 1965). Therefore, it is possible to follow its binding in the presence of different ligands, such as 8-MOP, to see if the binding of one ligand affects the binding of the other and in what fashion. As seen in Fig. 4, non-competitive inhibition of ethidium binding produces a Scatchard plot in which the slope (K) decreases in the presence of increasing amounts of 8-MOP, along with the change at the intercept on the abscissa (n). The results have been summarized in Table 1. Any change in K

and n for ethidium binding to DNA was not recorded above 0.0364 mM drug concentration. The changes in both K and n indicated the non-competitive inhibitory behavior of the ligand (8-MOP) on Et–DNA interaction (Howe-Grant et al., 1976).

3.4. Circular dichroism

As indicated in Fig. 5, the CD spectrum of DNA in the presence of ethidium bromide shows a special maximum band at 308 nm which is the characteristic one for intercalating agents (Gray et al., 1992). Also, the positive band at 275 nm and the increment in the negative band at 248 nm are other reasons for this kind of interaction (Gray et al., 1992). In this study, such type of peaks has not been recorded in the CD spectra of DNA in the presence of different concentrations of 8-MOP. However, some small changes observed in the spectra of DNA-8-MOP imply interaction with minor conformational changes in the DNA structure.

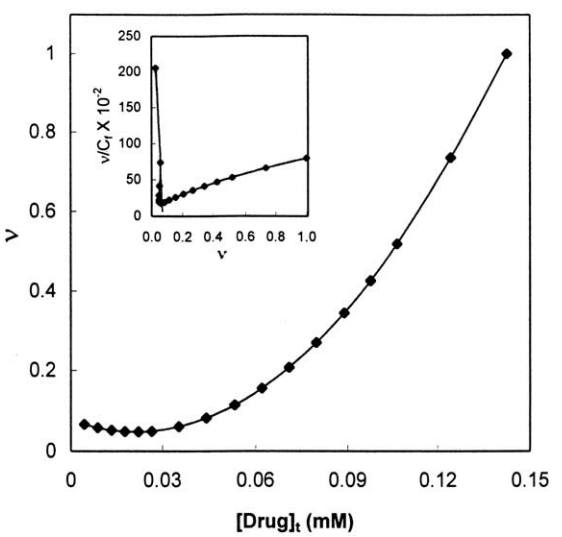


Fig. 3. The plot of binding isotherm as ν (the extent of ligand molecules bound per nucleotide, which is equal to the ratio of $[8\text{-MOP}]_{bound}/[DNA]_{total}$) against the total drug concentrations. (Inset) Scatchard plot for the binding of methoxalen to DNA, where ν is the binding ratio and c_f is the free drug concentration.

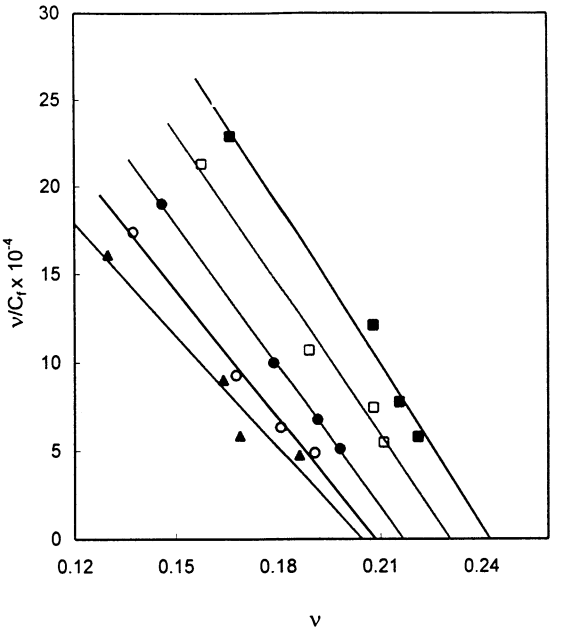


Fig. 4. The Scatchard plots of the binding of ethidium bromide (Et) to DNA in the absence and the presence of methoxalen obtained by fluorescence technique. The effect of five various concentrations of 8-MOP, ranging from 0.0091 to 0.0364 mM, was investigated on DNA–Et complex. The DNA concentration was 2.27 μMbp and the Et concentrations varied from 2.2 to 20.7 μM. (■) 0 mM 8-MOP; (□) 0.0091 mM 8-MOP; (●) 0.0228 mM 8-MOP; (○) 0.0318 mM 8-MOP; (▲) 0.0364 mM 8-MOP, which corresponds to $[8\text{-MOP}]_t/[DNA]$ molar ratio of 0, 2.0, 5.0, 7.0 and 8.0, respectively.

3.5. Temperature scanning spectrophotometry

Determination of the thermal denaturation temperature of DNA in the absence and presence of 8-MOP was carried out. The results for the samples with $[8\text{-MOP}]_t/[DNA]$ molar ratios be-

Table 1
Binding parameters for DNA–Et interaction in the absence and presence of different concentrations of 8-MOP at 25 °C

$[Drug]_t$ (mM)	K (M^{-1})	n
–	1.52×10^6	0.242
0.0091	1.44×10^6	0.230
0.0228	1.33×10^6	0.216
0.0318	1.20×10^6	0.208
0.0364	1.05×10^6	0.204

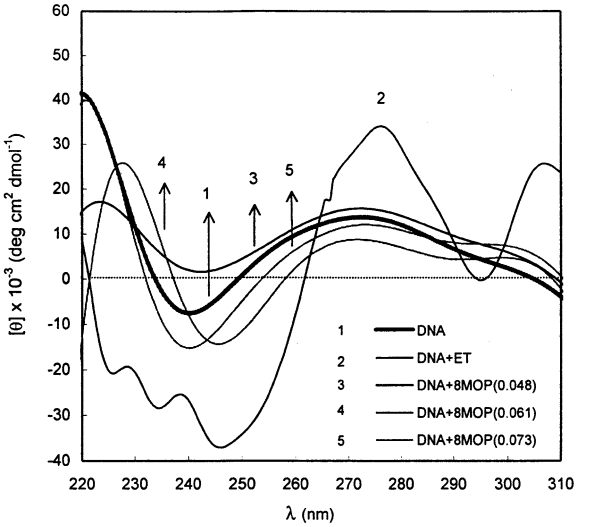


Fig. 5. The CD spectra of DNA in the absence (1) and in the presence of methoxalen (3–5) and of ethidium bromide (2). The plots have been shown in terms of molar ellipticity ([θ]) against the wavelengths of light. DNA concentration was 0.012 milli-molar base (mMb). Concentration of 8-MOP increases in the order of 0.048, 0.061 and 0.073 mM corresponding to [8-MOP]$_t$/[DNA] molar ratio of 4.0, 5.0 and 6.0, respectively for spectra 3–5. Ethidium bromide concentration was 18 μM.

tween 4 and 6 (data not shown) indicated ≈ 3 °C increase in the T_m of DNA.

3.6. Microcalorimetry

Fig. 6 shows the plot of enthalpy change (ΔH) obtained by calorimetric technique, against the total concentration ratio of drug per DNA. This plot represents a two-stage curve indicating a two-step process. The first stage is an endothermic process, which is followed by an exothermic one. As observed, there is a maximum at the [8-MOP]$_t$/[DNA] ratio of ≈ 2. The enthalpy determination after the ratio of ≈ 7.3 was not possible because of the strength changes at the baseline, indicating the major changes in the complex structure. For a better explanation about the enthalpy changes during the progress of the reaction, the plot of the ΔH_v ($\Delta H/v$, i.e. the enthalpy changes due to the binding of each mole of ligand per each mole of macromolecule) against v has been drawn (Fig. 7). It shows the endothermic process up to $v = 0.06$,

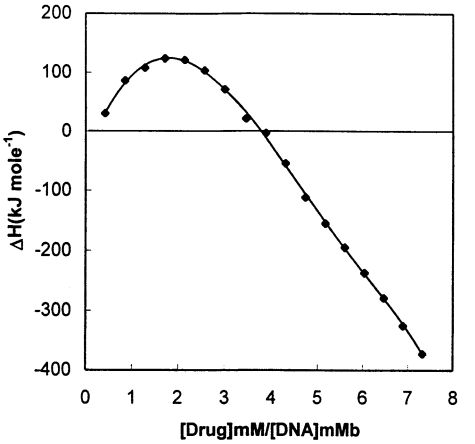


Fig. 6. The enthalpy change versus the molar ratio of [8-MOP]$_t$/[DNA]. The data were obtained from successive 90 μl injections of 0.115 mM methoxalen into a 1.5 ml solution of 0.016 milli-molar base (mMb) DNA. Titrant concentrations varied from 0.0065 to 0.062 mM corresponding to the [8-MOP]$_t$/[DNA] ratio from 0.43 to 7.3.

which indicates binding of ≈ 1 mol of 8-MOP to 17 mol nucleotides of DNA (near eight base pairs of DNA). Binding of more drug to DNA shows the exothermic process, which is followed by change in the sign of the enthalpy after the v of ≈ 0.12 ([8-MOP]$_t$/[DNA] molar ratio of ≈ 4) and

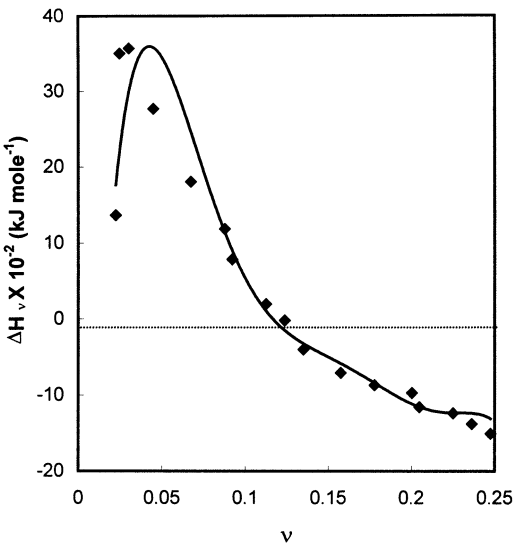


Fig. 7. The dependence of ΔH_v ($\Delta H_v = \Delta H/v$) on the v (the number of 8-MOP molecules bound per DNA nucleotide).

continue the same slope up to the v of 0.2 ([8-MOP]$_t$/[DNA] molar ratio of ≈ 6). Thereafter, the enthalpy changes are very slow, indicating the saturation of DNA with 8-MOP.

4. Discussion

Psoralens are one of the most important photochemical reactants for DNA and RNA structural analysis (Cimino et al., 1985). Although many studies have been made on the mechanism of psoralens, especially 8-methoxypsoralen–DNA interaction upon UV-irradiation (Isaacs et al., 1977; Dall'Acqua et al., 1979; Cimino et al., 1985; Yeung et al., 1988; Sastry et al., 1997), there is little information about the exact mechanism of their interaction in darkness.

Here, the calorimetric study of the 8-MOP–DNA interaction was performed. The plot of ΔH obtained by ITC versus the [8-MOP]$_t$/[DNA] molar ratio (Fig. 6) shows the two-stage curve with a maximum at the [8-MOP]$_t$/[DNA] ratio of ≈ 2. According to the Scatchard analysis of the fluorimetric data, at this ratio 1 mol of drug binds per each eight base pairs of DNA. It was previously reported that 8-MOP in low concentration binds to DNA intercalatively (Cole, 1970; Dall'Acqua et al., 1978; Straub et al., 1981). The intercalation is accompanied with some distortion in DNA structure and some DNA conformational relaxation (Saenger, 1984). It was also previously shown that the DNA unfolding is an endothermic process because of the exposition of the hydrophobic residues to the solvent (Bathaie et al., 1999). Thus, the binding of 8-MOP to DNA at lower concentrations induced partial unfolding for DNA through the intercalative mechanism, which is an endothermic process. By raising the drug concentration, the exothermic process is observed (Fig. 6). At the [8-MOP]$_t$/[DNA] ratio above 4, the enthalpy changes are accompanied by change in the sign and then reaches the steady state more than the [8-MOP]$_t$/[DNA] ratio of 6. In the concentration ratios above this, the precipitation is observed. Also, the spectrophotometric results show the decrement in the absorbance of DNA at 260 nm upon methoxalen addition and it reaches the steady state above the [8-MOP]$_t$/[DNA] molar ratio of 6. These results indicate some compaction in DNA upon interaction at high concentration of 8-MOP, which is the exothermic process (Bathaie et al., 1999) and DNA saturation at higher concentrations. The increase of ≈ 3 °C in the T_m of DNA upon methoxalen addition indicates that DNA becomes more stable. It is another affirmative reason for DNA compaction. The binding parameters for 8-MOP–DNA interaction are calculated according to the data obtained from the quenching of methoxalen fluorescence emission by DNA and Scatchard analysis (Fig. 2). The Scatchard plot shows a two-step curve indicating the existence of two sets of binding sites (Bordbar et al., 1996) on DNA for 8-MOP. The first set, which is considered with the intercalative mechanism, has the $K = 5.02 \times 10^5$ M^{-1} and $n = 0.06$ (i.e. 1 mol of drug binds per near each 8 bp of DNA). In comparison, Gupta and Ali (1984) had reported the $K = 7.1 \times 10^5$ M^{-1}. For investigation of the mechanism of interaction at the second set of binding sites, we have utilized the Scatchard analysis of the Et–DNA complex in the presence of different concentrations of methoxalen. The increased emission of Et due to the binding to DNA is quenched by 8-MOP. The Scatchard plots for Et–DNA complex in the absence and the presence of 8-MOP (Fig. 4) show the non-competitive behavior for quenching. Such behavior was reported previously by different ligands, including some porphyrins (Fiel, 1989; Pasternak et al., 1991), several platinum and palladium complexes (Howe-Grant et al., 1976) and methylviologen (Fromherz and Rieger, 1986). The proposed mechanism for this behavior is the non-displacement based quenching due to the enhanced energy transfer, either from excited ethidium to an acceptor or from a donor to an excited ethidium acceptor (Pasternak et al., 1991). The CD spectrum of DNA in the presence of Et shows a characteristic peak at 308 nm, which is accompanied by a broad positive band at 275 nm and an increase in the negative peak at 248 nm. These findings are the reasons for the intercalative mechanism of Et binding to DNA (Gray et al., 1992). Such characteristic changes were not observed in DNA spectrum upon interaction with 8-MOP at used higher

$[8\text{-MOP}]_t/[DNA]$ molar ratios (4–6). Therefore, 8-MOP–DNA binding does not involve the intercalation at higher concentrations of drug while it binds to the outside of DNA, possibly through the minor groove.

5. Conclusion

It is concluded from the present results that there are two different mechanisms for methoxalen binding to DNA. At low drug concentration it binds intercalatively, but at high drug concentration it binds to the outside of DNA. At higher concentrations, DNA compaction occurs, which then causes DNA precipitation.

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