Structural Analysis of DNA-Chlorophyll Complexes by Fourier Transform Infrared Difference Spectroscopy

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ABSTRACT Porphyrins and metalloporphyrins are strong DNA binders. Some of these compounds have been used for radiation sensitization therapy of cancer and are targeted to interact with cellular DNA. This study was designed to examine the interaction of calf thymus DNA with chlorophyll *a* (CHL) in aqueous solution at physiological pH with CHL/DNA(phosphate) ratios (*r*) of 1/160, 1/80, 1/40, 1/20, 1/10, and 1/5. Fourier transform infrared (FTIR) difference spectroscopy was used to characterize the nature of DNA-pigment interactions and to establish correlations between spectral changes and the CHL binding mode, binding constant, sequence selectivity, DNA secondary structure, and structural variations of DNA-CHL complexes in aqueous solution. Spectroscopic results showed that CHL is an external DNA binder with no affinity for DNA intercalation. At low pigment concentration ($r = 1/160$, 1/80, and 1/40), there are two major binding sites for CHL on DNA duplex: 1) Mg-PO_2 and 2) Mg-N7 (guanine) with an overall binding constant of $K = 1.13 \times 10^4\ M^{-1}$. The pigment distributions are 60% with the backbone PO_2 group and 20% with the G-C base pairs. The chlorophyll interaction is associated with a major reduction of B-DNA structure in favor of A-DNA. At high chlorophyll content ($r = 1/10$), helix opening occurs, with major spectral alterations of the G-C and A-T bases. At high chlorophyll concentration (1/5), pigment aggregation is observed, which does not favor CHL-DNA complexation.

INTRODUCTION

Porphyrins and their metal derivatives are strong DNA binders, with association constants of $10^5\ M^{-1}$ to $10^6\ M^{-1}$ (Lipscomb et al., 1996; Sari et al., 1990; Pasternack et al., 1986; Anantha et al., 1998). Some of these compounds have been used for radiation sensitization therapy of cancer and are targeted to interact with cellular DNA (Hill, 1991). On the other hand, diet has been shown to be an important determinant of human cancer risk (Doll, 1990; Doll and Peto, 1981). Several chemical constituents in fruits and vegetables have now been purified and shown to protect against carcinogenesis in experimental animals, and some of these compounds are now in clinical trials (Farber, 1982; Wattenberg, 1990; Hayatus et al., 1988; Dragsted et al., 1993). Chlorophyll (CHL) (structure 1) and its derivatives exert profound antimutagenic behavior against a wide range of potential human carcinogens (Lai et al., 1980; Kimm et al., 1982; Kimm and Park, 1982). Chlorophyllin, a food-grade derivative of chlorophyll, has been used historically in the treatment of several human conditions, with no evidence of human toxicity (Ong et al., 1986; Harrison et al., 1954; Young and Beregi, 1980), and recently it has been used as a potent inhibitor of aflatoxin B_1 hepatocarcinogenesis in trout (Breinholt et al., 1995). It has been suggested that the antimutagenic activity of chlorophyllin comes from its strong complexation with parent mutagens or their intermediates, scavenging of free radicals and active oxygen species, and suppression or interference with metabolic activation by specific cytochrome (P-450) and other metabolizing enzymes (Newmark, 1984; Arimoto et al., 1980; Dashwood et al., 1991; Romert et al., 1992). Chlorophyllin was also shown in vivo to efficiently inhibit precarcinogenic target organ DNA adduction by aflatoxin B_1 in trout (Breinholt et al., 1995) and 2-amino-3-methylimidazol [4,5-f]quinoline in rats (Dashwood, 1992).

Because the main target of metalloporphyrins can involve DNA or DNA adducts, the interaction of CHL with DNA has major biochemical importance. The present study is designed to investigate DNA-CHL complexation in vitro and to provide structural information regarding the pigment binding mode, association constant, sequence preference, and DNA secondary structure, using infrared spectroscopy. To our knowledge, our structural information provides the first spectroscopic evidence regarding DNA-CHL interaction and should help to elucidate the nature of this biologically important complex formation. Recently, we have used vibrational spectroscopy (infrared and Raman) for the structural characterization of several DNA-drug (Neault and Tajmir-Riahi, 1996; Neault et al., 1995, 1996), DNA-cation (Tajmir-Riahi et al., 1995a,b), and protein complexes (Ahmed et al., 1995). We believe that Fourier transform infrared (FTIR) difference spectroscopy can be also applied here to characterize the nature of the DNA-pigment interaction and provide structural information on the DNA-CHL complexes formed in aqueous solution.

In this work, FTIR difference spectroscopy was applied to study the interaction between calf thymus DNA and chlorophyll *a* in aqueous solution at physiological pH with CHL/DNA(P) molar ratios of 1/160 to 1/5. Structural analyses regarding the pigment-binding site, binding constant,

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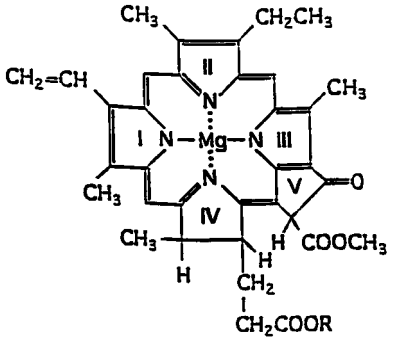

Scheme

sequence dependence, and biopolymer secondary structure are provided. Furthermore, comparisons are made between the DNA-CHL complexes and those of the corresponding metalloporphyrin-DNA adducts, and the results are reported here.

MATERIALS AND METHODS

Materials

Highly polymerized type I calf thymus DNA sodium salt (7% Na content) was purchased from Sigma Chemical Co. and was deproteinated by the addition of $CHCl_3$ and isoamyl alcohol in NaCl solution. Crystalline chlorophyll *a* was from Sigma Chemical Co. and was used without further purification. Other chemicals were of reagent grade.

Preparation of stock solutions

Sodium-DNA was dissolved to 2% w/w (0.05 M DNA(phosphate)) in 0.1 M NaCl and 1 mM sodium cacodylate (pH 7.30) at 5°C for 24 h with occasional stirring to ensure the formation of a homogeneous solution. The appropriate amount of chlorophyll *a* (0.15–5 mM) was prepared in ethanol (chlorophyll is not soluble in H_2O or D_2O) and added dropwise to DNA solution to attain desired CHL/DNA(P) molar ratios of 1/160, 1/80, 1/40, 1/20, 1/10, and 1/5 at a final DNA concentration of 1% w/w or 0.025 M DNA(phosphate). The pH solution was adjusted to 7.30–6.80, using NaOH solution. The infrared spectra were recorded 3 h after mixing of CHL and DNA solutions. The infrared spectra of DNA-CHL complexes with $r > 1/5$ could not be recorded as a solution because of solid gel formation.

Physical measurements

Infrared spectra were recorded on a Bomem DA3–0.02 FTIR spectrometer equipped with a nitrogen-cooled HgCdTe detector and KBr beam splitter. The solution spectra are taken using AgBr windows with a resolution of 2–4 cm^{-1} and 100–500 scans. The sample preparation and spectral measurements are carried out under green light (to avoid photodegradation of chlorophyll in CHL-DNA complexes). Each set of infrared spectra was taken (three times) on three identical samples with the same DNA and pigment concentrations. The water subtraction was carried out with 0.1 M NaCl solution as a reference at pH 6.5–7.5 (Alex and Dupuis, 1989). A good water subtraction is achieved as shown by a flat baseline around 2200 cm^{-1}, where the water combination mode is located. This method is a rough estimate but removes the water content in a satisfactory way. Because of the insolubility of chlorophyll in D_2O, we did not run the same experiments in D_2O solution. The difference spectra [(DNA solution + CHL solution) − (DNA solution)] are produced, using a sharp DNA band at 968 cm^{-1} as internal reference. This band, due to deoxyribose C-C stretching vibrations, exhibits no spectral changes (shifting or intensity variations) on CHL-DNA complexation, and it is cancelled upon spectral subtraction. The spectra are smoothed with a Savitzky-Golay procedure (Alex and Dupuis, 1989). The intensity ratios of several DNA in-plane vibrations related to A-T and G-C base pairs and the PO_2 stretchings are measured (with respect to the reference band at 968 cm^{-1}) as a function of CHL concentration with an error of ±3%. These intensity ratio measurements are used to quantify the amount of CHL binding to the backbone PO_2 group and DNA bases.

RESULTS AND DISCUSSION

DNA-CHL complexes

At low chlorophyll concentration ($r = 1/160$, 1/80 and 1/40), CHL binds directly to the backbone PO_2 group and indirectly (via H_2O) to the N-7 site of the guanine bases through the central Mg cation. Evidence for direct cation-phosphate coordination is concluded from the major intensity increase (50%) of the PO_2 antisymmetical stretching band at 1222 cm^{-1}, together with the shift of this vibration toward a lower frequency at 1220 cm^{-1} (Figs. 1 and 2). Similarly, the guanine band at 1717 cm^{-1} (Alex and Dupuis, 1989; DiRico et al., 1985; Spiro, 1987; Loprete and Hartman, 1993; Starikov et al., 1991; Keller and Hartman, 1986; Prescot et al., 1984; Taillandier et al., 1985) gains in intensity (25%) and shifts toward a lower frequency at 1715 cm^{-1}, which is due to an indirect Mg-N-7 interaction via a H_2O molecule (Figs. 1 and 2). The bands at 1663 cm^{-1} (mainly thymine) (Alex and Dupuis, 1989; DiRico et al., 1985; Spiro, 1987; Loprete and Hartman, 1993; Starikov et al., 1991; Keller and Hartman, 1986; Prescot et al., 1984; Taillandier et al., 1985) showed an increase in intensity (30%) with no spectral shifting upon CHL interaction, whereas the bands at 1609 (adenine) and 1485 cm^{-1} (mainly cytosine) exhibited a minor increase in intensity with no frequency shift in the spectra of the CHL-DNA complexes (Figs. 1 and 2, $r = 1/160$). The observed spectral changes show a direct Mg-PO_2 binding with an indirect Mg-H_2O···N-7 coordination to the guanine bases. However, a minor indirect cation coordination via H_2O to the thymine O-2 atom can also be included, whereas metal ion binding to the adenine and cytosine bases is negligible. It is worth mentioning that at this stage of complexation, the Mg-DNA binding sites are not involved in the Watson-Crick hydrogen bonding network, and therefore this type of complexation does not bring about helix destabilization. It should be noted that the direct Mg-PO_2 coordination and indirect Mg-N-7 (guanine) interaction through H_2O are found in the crystal structure of d(CpGpCpGpCpG) oligonucleotide, stabilizing left-handed Z-DNA conformation (Gessner et al., 1985). On the basis of the spectroscopic results, the Mg cation binding to the backbone PO_2 and the guanine N-7 sites is also suggested for the MgATP complexes with calf thymus DNA in hydrated films (Bhattacharyya et al., 1988). Raman spectroscopic studies also showed direct Mg-PO_2 binding for the Mg-DNA complexes in aqueous solution (Langlais et al., 1990). On the basis of the FTIR spectroscopy, direct and indirect Mg cation binding to the phosphate group and

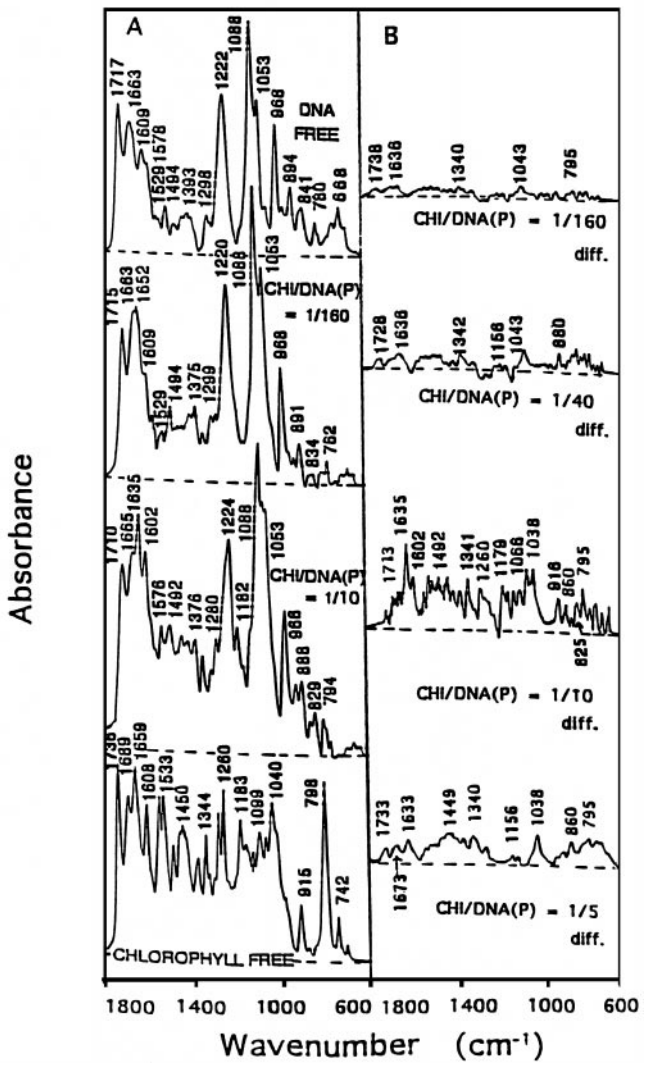

FIGURE 1 FTIR spectra (*A*) and difference spectra [(DNA solution + CHL solution) − (DNA solution)] (*B*) for the uncomplexed calf thymus DNA and its chlorophyll (CHL) adducts in aqueous solution at pH 6.8–7.3 with different CHL/DNA(P) molar ratios in the region of 1800–600 cm^{-1} (the baselines are shown as *broken lines*).

the base N-7 atom of guanine or adenine was found in the solid-state and solution structures of Mg-guanosine-5′-monophosphate (Mg-GMP), Mg-deoxyguanosine-5′-monophosphate (Mg-dGMP), and Mg-adenosine-5′-monophosphate (Mg-AMP) complexes (Tajmir-Riahi, 1990a,b, 1991).

The calculation of the binding constants was carried out as reported for other drug complexes with DNA, RNA, and mononucleotides (Muller and Crothers, 1975; Tuite and Kelly, 1995; Tuite and Norden, 1994; Neault and Tajmir-Riahi, 1997; Tajmir-Riahi et al., 1988). Assuming that a major complexation occurs between the CHL and DNA molecule, the following equations can be established:

$$\text{DNA} + \text{CHL} \Leftrightarrow \text{DNA: CHL} \quad K \tag{1}$$

$$K = \frac{[\text{DNA:CHL}]}{[\text{DNA}][\text{CHL}]} \tag{2}$$

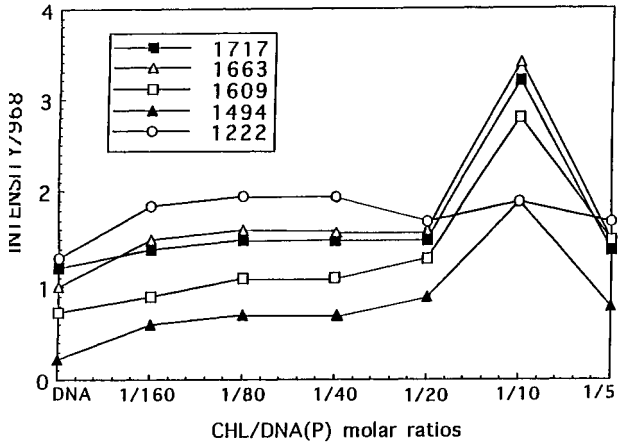

FIGURE 2 Intensity ratio variations for several DNA in-plane vibrations at 1717 (G, T), 1663 (T, G,A, C), 1609 (A), 1494 (C, G), and 1222 cm^{-1} (PO_2 stretch) as a function of chlorophyll (CHL) concentration (different CHL/DNA(P) molar ratios).

Because pigment interactions occur mainly through the backbone PO_2 group and the guanine bases, the relative intensity variations of the bands at 1222 cm^{-1} (PO_2 stretch) and 1717 cm^{-1} (mainly G) were calculated for each CHL concentration.

The double-reciprocal plot of $1/(I - I_0)$ versus $1/(L)$ is linear, and the binding constant (K) is estimated from the ratio of the intercept to the slope. I_0 is the initial relative intensity of the specific DNA absorption band, and I is the relative intensity at different CHL concentrations (L). The overall binding constant for CHL-DNA complexes is estimated to be $K = 1.13 \times 10^4\ M^{-1}$. Similar intensity ratio measurements were used as a function of pigment concentration to estimate the K(P) for the phosphate and the K(G) for the guanine bases. The calculated binding constants were $K(P) = 2.90 \times 10^4\ M^{-1}$ and $K(G) = 4.40 \times 10^3\ M^{-1}$. Similar methods, based on the intensity ratio variations of the Raman and infrared vibrational frequencies, have been used to determine the binding constants of diethylstilbestrol (intercalating drug) binding to polynucleotides (Neault and Tajmir-Riahi, 1997) and the CH_3Hg^+ cation binding to mononucleotides (Tajmir-Riahi et al., 1988).

The calculated binding constants for the CHL interaction with the backbone phosphate groups, $K(P) = 2.90 \times 10^4\ M^{-1}$ and $K(G) = 4.40 \times 10^3\ M^{-1}$, provide additional evidence regarding the backbone PO_2 group as a primary target and the guanine N-7 atom as the secondary site for the Mg cation coordination. The value obtained for the binding constant is also comparable with those of the hydrated Mg cation coordination to DNA and RNA in aqueous solution (Izatt et al., 1971; Danchin, 1972). At a chlorophyll concentration of 2.5×10^{-4} M, the distributions of the bound CHL are ~60% with the backbone PO_2 groups and ~20% with the G-C base pairs (Fig. 3).

A strong band at 1652 cm^{-1} in the spectrum of the DNA-CHL adduct (absent from the spectrum of the free DNA) was related to the CHL in-plane vibration at 1659

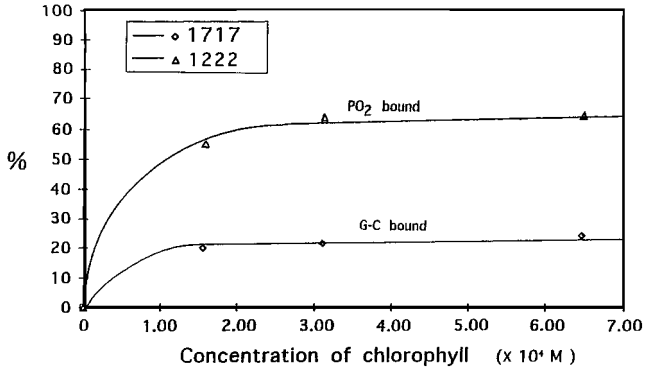

FIGURE 3 Calculated distributions of the chlorophyll bound to the backbone PO_2 group and G-C base pairs in aqueous solution with a DNA concentration of 0.025 M (phosphate), as a function of CHL concentration (M) based on a binding constant of $K = 1.13 \times 10^4\ M^{-1}$, for the bands at 1717 cm^{-1} (G) and 1222 cm^{-1} (PO_2).

cm^{-1} (Fujiwara and Tasumi, 1986; Bardwell and Dignam, 1987) that shifted to lower frequencies upon DNA complexation (Fig. 1, $r = 1/160$).

The Mg cation is four-coordinate in the CHL complex, when it is dissolved in nonpolar solvents, whereas in aqueous solution the Mg coordination extends to five or six by cation interaction with H_2O or other oxygen atoms from neighboring CHL molecules (as axial ligands), forming dimers or polymers in the aggregated state (Fujiwara and Tasumi, 1986; Bardwell and Dignam, 1987). Upon DNA interaction, the axial ligand is replaced by the backbone PO_2 group (directly) or by the guanine base N-7 atom (indirectly via H_2O), because the major affinity of the Mg cation toward the backbone phosphate binding is well demonstrated (Eichhorn and Shin, 1968). It is important to note that the presence of the axial ligands on the Mg cation do not favor CHL intercalation into the A-T or G-C base pairs (because of the steric blockage), and complex formation is limited to the outside binding mode.

At $r = 1/10$, a major increase in the intensities of several DNA in-plane vibrations at 1717 cm^{-1} (G, T), 1663 cm^{-1} (T, A, G, C), 1609 cm^{-1} (A), 1494 cm^{-1} (C, G), and 1222 cm^{-1} (PO_2 stretch) was observed as a result of partial helix opening (Fig. 2). The partial helix melting provides additional binding sites for CHL complexation. Evidence for this was obtained from a major intensity increase and frequency shift of the bands at 1663 cm^{-1} to 1665 cm^{-1}, 1609 cm^{-1} to 1602 cm^{-1}, and 1494 cm^{-1} to 1492 cm^{-1} in the spectra of the CHL-DNA complexes formed at high pigment concentration (Fig. 1 *A*). The spectral changes observed were due to the participation of the G-C and A-T base pairs on CHL complex formation. The positive features at 1713, 1602, 1492, 1341, 1179, and 1066 cm^{-1}, in the difference spectra of the CHL-DNA complexes formed at $r = 1/10$, are due to a major increase in the intensity of DNA vibrations, upon helix destabilization (Fig. 1 *B*). Similar increases in the intensity of several DNA in-plane vibrations were observed in the presence of high DES (diethylstilbestrol) concentration, which was attributed to a partial helix opening and DNA melting, upon drug complexation (Neault and Tajmir-Riahi, 1996). The interaction of several cations with DNA also results in a major increase in the intensity of DNA vibrations due to the cation coordination and helix destabilization (Tajmir-Riahi et al., 1995a,b). At this stage, not only is the Mg cation the main DNA binder, but an extended H-bonding network between the biopolymer donor atoms and the chlorophyll polar groups can also be detected during complexation. Evidence for this comes from major spectral shifts of the chlorophyll vibrations at 1736, 1659, and 1608 cm^{-1} related to the pigment exocyclic COO, C═O, and C-O stretching vibrations (Fujiwara and Tasumi, 1986; Bardwell and Dignam, 1987), upon DNA interaction (Fig. 1).

It is important to note that chlorophyll complexation with DNA leads to a partial reduction of B-DNA structure in favor of A-DNA. Evidence for this conformational transition comes from the shifts of the DNA marker bands at 1717 (G, T) to 1710 cm^{-1}, 1222 (PO_2) to 1224 cm^{-1}, and 841 (phosphodiester) to 829 cm^{-1} (Fig. 1). The shifts of these conformational indicators (Loprete and Hartman, 1993; Keller and Hartman, 1986; Taillandier et al., 1985), together with the emergence of a new band at 860 cm^{-1}, are related to the reduction of the B-DNA structure and the formation of A-DNA, upon CHL interaction (Fig. 1, 1/10). The marker infrared bands for B-DNA structure are positioned at 1717 cm^{-1} (G, C), 1222 cm^{-1} (PO_2 stretch) and 840–836 cm^{-1} (sugar-phosphate) (Loprete and Hartman, 1993; Starikov et al., 1991; Keller and Hartman, 1986). When B-to-A transition occurs, these marker bands are shifted to 1710–1700 cm^{-1}, 1240–1225 cm^{-1} and 825–800 cm^{-1}, respectively, and a new band appears at ~870–860 cm^{-1} (Loprete and Hartman, 1993; Keller and Hartman, 1986; Taillandier et al., 1985).

At high CHL concentrations ($r = 1/5$), pigment aggregation occurs, which does not favor chlorophyll-DNA interaction. Evidence for this comes from a major loss of the intensity of DNA vibrations at 1717, 1663, 1609, 1494, and 1222 cm^{-1} (Figs. 1 *B* and 2). The spectral changes observed are due to a partial dissociation of the CHL-DNA complexes in favor of pigment-pigment interaction at high CHL conentration. The aggregation of the porphyrins and their metal derivatives is known, and the effects of pigment polymerization on DNA complexation are well investigated (Pasternack et al., 1983a,b). Similarly, the aggregation of chlorophyll through its central Mg cation is reported (Oksanen et al., 1996, and references therein). The Mg cation expands its coordination from 4 to 5 or 6 by interaction with solvent molecules and the porphyrin ring external donor atoms (Oksanen et al., 1996, and references therein). It should be noted that the difference spectrum obtained at high pigment concentration ($r = 1/5$) shows marked similarities to those of the CHL-DNA complexes formed with low pigment contents (Fig. 1, 1/40). This indicates that the CHL-DNA complexation is mainly limited to the low pigment concentrations, where CHL aggregation does not occur.

Additional evidence regarding the DNA-CHL complexation at low pigment concentrations comes from the major spectral shifts of several chlorophyll vibrations at 1736 cm^{-1} (C═O stretch), 1689 cm^{-1} (C═O stretch), 1659 cm^{-1} (C═O and C═C stretch), and 1608 cm^{-1} (C═N and C═C stretches) (Fujiwara and Tasumi, 1986; Bardwell and Dignam, 1987) toward lower frequencies on DNA interaction (Fig. 2). Other absorption bands at 1260, 1183, 1040, and 798 cm^{-1}, in the free pigment spectrum are related to the chlorophyll C-O and C-C stretches (Fujiwara and Tasumi, 1986; Bardwell and Dignam, 1987) that exhibit shifts on DNA complexation (Fig. 1). The observed spectral changes of the CHL vibrational frequencies are due to the participation of Mg cation in DNA binding and the rearrangements of the pigment hydrogen bonding network in the CHL-DNA complexes. Several positive features at 1733, 1673, 1633, 1449, 1038, and 795 in the difference spectra of the CHL-DNA complexes are from chlorophyll vibrational frequencies, and they are not related to DNA vibrations (Fig. 1 *B*, $r = 1/5$).

Comparisons with Other Metalloporphyrin-DNA Complexes

Previous studies have mainly been concerned with the interaction of polynucleotides with water-soluble porphyrins and metalloporphyrin complexes (Lipscomb et al., 1996; Sari et al., 1990; Pasternack et al., 1986; Anantha et al., 1998; Kelly et al., 1985; Hudson et al., 1992; Brun and Harriman, 1994; Wheeler et al., 1995; Kruglik et al., 1995), whereas little is known about DNA complexation with water-insoluble porphyrins and their metal derivatives. It has been suggested that planer four-coordinate metalloporphyrins are selectively intercalated into the G-C rich region, whereas porphyrins having cations with axial coordination (five- or six-coordinate) form outside binding with the A-T or G-C donor atoms (Wheeler et al., 1995; Kruglik et al., 1995). Various spectroscopic methods such as UV-visible, CD, fluorescence, electron paramagnetic resonance, NMR, and Raman have been applied to characterization of the nature of water-soluble porphyrins and their metal derivatives with synthetic and native DNAs (Hudson et al., 1992; Brun and Harriman, 1994; Wheeler et al., 1995; Kruglik et al., 1995). Raman spectroscopic results have shown that when porphyrin or its metal derivative intercalates into the A-T or G-C regions, certain DNA vibrations related to A and T or G and C bases are affected, and spectral changes such as band shift or intensity variations are observed (Wheeler et al., 1995; Kruglik et al., 1995).

The spectroscopic results presented here for the first time clearly show that at low pigment content, the CHL interaction with DNA is through outside binding with major spectral alterations (intensity variations and shifts) of the backbone PO_2 and G-C vibrations. The primary target of the CHL is the backbone PO_2 group and the N-7 sites of the guanine bases, with an overall binding constant of $K = 1.13 \times 10^4\ M^{-1}$. The CHL-DNA interaction is accompanied by a major reduction of the B-DNA structure in favor of A-DNA. At high CHL concentration, a partial helix opening occurs, and the pigment aggregation does not favor CHL-DNA complexation.

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