double bond pushes both atoms toward higher positive charges by fairly comparable amounts, so that all atoms take the charge brought by the $2H^+$. A similar democratic sharing of the charge also occurs from diprotonation of bridged C_2H_4. For Si_2H_4, the net charges are not exactly modified this way. Under diprotonation, the hydrogen atoms of the Si═Si double bond become positively charged and the part of the global charge taken by these hydrogens is about the same as that for C_2H_4. On the other hand, the two protons added to the Si═Si double bond do not contribute to the sharing of the double positive charge, as in the preceding case, since they take a formal negative charge. Therefore, the remainder of positive charge is supported by the silicon atoms. When the Si_2H_4 double bridge is diprotonated, the H_b and H_t hydrogens do not equally share the charge, as was the case for C_2H_4. Now, the bridging hydrogens H_b are little affected and keep a negative charge while the H_t and Si atoms undertake most of the positive charge. The changes brought by the protonation of the lone pairs extracyclic to the double bridge are therefore different in C_2H_4 and Si_2H_4. The structure of the double bridge remains rather unchanged in $Si_2H_6^{2+}$ while it is more perturbed in $C_2H_6^{2+}$. This is also confirmed by the H_b charges calculated from the CASSCF + NAMO procedure. As mentioned in our previous paper,[4] such charges may be very different from the SCF Mulliken ones. However, they are quite in line with the above-discussed trends since the CASSCF charge on H_b is increased more upon protonation for C_2H_4 than it is for Si_2H_4 (see Table XII).

Registry No. B_2H_6, 19287-45-7; Ga_2H_6, 12140-58-8; C_2H_4, 74-85-1; Si_2H_4, 15435-77-5; Ge_2H_4, 82323-93-1; Sn_2H_4, 86041-63-6; Pb_2H_4, 90176-62-8; $C_2H_6^{2+}$, 136061-29-5; $Si_2H_6^{2+}$, 136061-28-4; cyclopropenyl cation, 26810-74-2; allyl cation, 1724-44-3.

Photochemically Induced Dynamic Nuclear Polarization Studies of Oligonucleotide Duplexes

Masato Katahira,[1a,b] Ritsuko Sakaguchi-Katahira,[1a,c] Fumiaki Hayashi,[1a,d] Seiichi Uesugi,[1e] and Yoshimasa Kyogoku*,[1a]

Contribution from the Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan, and Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan.
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Abstract: The application of the photochemically induced dynamic nuclear polarization (photo-CIDNP) technique to structural studies of nucleic acids has been attempted. The CIDNP signals of double-stranded nucleic acids were observed for the first time. The CIDNP signals were observed clearly for A and B form oligonucleotides examined, $r(CGCGCG)_2$ and $d(CGCGCG)_2$ in 0.15 M NaCl, respectively, while those for Z form oligonucleotides examined, $r(CGCGCG)_2$ and $d(CGCGCG)_2$ in saturated $NaClO_4$, were very weak. The central two guanosine residues of $d(GGGGCCCC)_2$ give an intense CIDNP signal, which disappears when the drug, chromomycin, binds to the minor groove of this oligonucleotide. The most likely explanation for this result is that chromomycin bound to the minor groove interferes with the access of a flavin molecule to the base and prevents the photoreaction indispensable for the appearance of the CIDNP signal. The correlation between the unusual cutting of some oligonucleotides by the drug, bleomycin, and their CIDNP signals was also found. The results presented here indicate the possible application of the photo-CIDNP technique to structural studies of nucleic acids.

The photo-CIDNP technique is a very unique ^{1}H NMR spectroscopic technique and has been applied successfully to proteins to study their surface structure and their interactions with ligands (see Kaptein[2] for a review). On the other hand, its application to nucleic acids has been limited to mononucleotides,[3-5] dinucleotides[4,6] and the single-stranded region of tRNA.[7] It has been believed that this technique is only useful for detecting single-stranded nucleic acids, i.e., not for structural studies of double-stranded nucleic acids because CIDNP signals have not been observed for double-stranded nucleic acids such as $d(CGCG)_2$[8] and $r(AGCU)_2$.[5] Here, however, we observed the CIDNP signals of double-stranded nucleic acids for the first time, and the relationship between the appearance of the CIDNP signals and the structures of nucleic acids has been studied.

Previous studies showed that the base protons of purine bases, guanine and adenine, give rise to the CIDNP signals.[3,5] The presence of the guanine, however, suppresses the CIDNP signal of the adenine.[5] Therefore, we concentrated on the CIDNP signal of the base proton of the guanine, the H8 proton. First, in order to investigate the structure-dependency of the CIDNP signals of double-stranded nucleic acids, the CIDNP signals of the following oligonucleotides were examined: $r(CGCGCG)_2$ in 0.15 M NaCl (A form), $d(CGCGCG)_2$ in 0.15 M NaCl (B form), and $r(CGCGCG)_2$ and $d(CGCGCG)_2$ in saturated $NaClO_4$ (Z form). Secondly, the CIDNP signals of $d(GGGGCCCC)_2$ and the chromomycin–$d(GGGGCCCC)_2$ complex, where an antitumor drug, chromomycin, is bound to the minor groove of this oligo-

* Correspondence should be addressed to: Professor Yoshimasa Kyogoku, Institute for Protein Research, Osaka University, 3-2, Yamadaoka, Suita, Osaka 565, Japan. Telephone: 81(Japan)-68779725; Fax: 81(Japan)-68762533.

(1) (a) Institute for Protein Research, Osaka University. (b) Present address: Department of Chemistry, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands. (c) Present address: International Research Laboratories, CIBA-GEIGY JAPAN, Miyuki-cho, Takarazuka, Hyogo 665, Japan. (d) Present address: Research Laboratory, Shionogi Pharmaceutical Company, Sagisu, Fukushima-ku, Osaka 553, Japan. (e) Faculty of Pharmaceutical Sciences, Osaka University.

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nucleotide,[9] were compared, and the effect on the CIDNP signal of interference with the access of a flavin molecule to the nucleic acids by chromomycin was studied. Thirdly, the CIDNP signals of $d(CCCCAGCTGGGG)_2$ (BL-1) and $d(GGGGAGCTCCCC)_2$ (BL-2) were compared. The antitumor drug, bleomycin, usually cuts oligonucleotides at GpC or GpT sites.[10] BL-1 is cut at a usual cutting site, GpC, while BL-2 is cut at an unusual site, GpA, predominantly, although it has a usual cutting site, GpC (Uesugi et al., unpublished results). The interesting correlation between the unusual cutting of BL-2 and its CIDNP signal is presented.

Materials and Methods

All oligonucleotides were synthesized and purified as reported elsewhere.[11,12] Chromomycin was purchased from Sigma Co. 3-(Carboxymethyl)lumiflavin (flavin I) was synthesized from lumiflavin purchased from Sigma Co.[13] Each lyophilized oligonucleotide was dissolved in 0.5 mL of D_2O containing 10 mM Na phosphate buffer (pH 7.0), 0.4 mM flavin I, and 0.15 M NaCl (for the A and B forms) or 0.15 M NaCl and saturated $NaClO_4$ (for the Z forms) to a concentration of 1 mM strands. DSS was used as an internal chemical shift reference.

One-dimensional normal and photo-CIDNP spectra were recorded on a JEOL GX-500 NMR spectrometer as reported elsewhere.[14] The laser power for irradiation was 0.8 W, and the irradiation time was 300 ms. The repetition delay was 5 s and 32 free induction decays were accumulated. No significant flavin bleaching was detected visually under the present experimental conditions. Subtraction of the dark and light spectra yielded a photo-CIDNP difference spectrum.

Results

The numbering of the residues of the oligonucleotides is as follows:

```
 1 2 3 4 5 6     1 2 3 4 5 6     1 2 3 4 5 6 7 8
rC G C G C G,   dC G C G C G,   dG G G G C C C C,
 1 2 3 4 5 6 7 8 9 10 11 12
dC C C C A G C T G G G G (BL-1) and
 1 2 3 4 5 6 7 8 9 10 11 12
dG G G G A G C T C C C C (BL-2)
```

Photo-CIDNP Spectra of A, B, and Z Form Oligonucleotides. As a representative of an A form oligonucleotide, $r(CGCGCG)_2$ in 0.15 M NaCl was selected. It has been confirmed that r-$(CGCGCG)_2$ takes on the A form under these conditions,[12,15,16] and the assignment of proton resonances has been performed.[15,16] CD and UV temperature profiles indicated that its melting temperature is 50 °C even at the very low concentration of 0.02 mM strands.[12] These profiles also indicate that the amount of melted single strands is negligible below 20 °C, even in the case of tetranucleotide $r(CGCG)_2$ at a concentration of 3 mM strands. We studied the chemical shift–temperature profile of base protons of $r(CGCGCG)_2$, which confirmed that the amount of melted single strands is completely negligible below at least 25 °C with a concentration of 1 mM strands, with which the photo-CIDNP experiment was carried out (data not shown).

As a representative of a B form oligonucleotide, $d(CGCGCG)_2$ in 0.15 M NaCl was selected. It has been confirmed that d-$(CGCGCG)_2$ takes on the B form under these conditions,[16,17] and the assignment of proton resonances has been performed.[16] CD and UV temperature profiles indicated that even with a low concentration of 0.2 mM strands the melting temperature is 52.5 °C and that the amount of melted single strands is completely negligible below 25 °C.[11]

As representatives of Z form oligonucleotides, $r(CGCGCG)_2$ and $d(CGCGCG)_2$ in saturated $NaClO_4$ were selected. It has been confirmed that nearly all (~90%) $r(CGCGCG)_2$ in 6 M $NaClO_4$ takes on the Z form,[18,19] and the assignment of proton resonances has been performed.[19] We confirmed that it takes on the Z form on detection of the syn conformation of guanosines. In Z form oligonucleotides, guanosines take on the syn conformation around a glycosyl bond, and in that case the intraresidue distance between H8 and H1′ protons is very short (~2.6 Å), while it is much longer (~3.8 Å) for the anti conformation observed in the A and B form oligonucleotides.[20] On irradiation of the resonances indicated by asterisks in Figure 1c (upper), which are assigned to the H8 protons of guanosines,[19] strong NOEs to H1′ protons comparable to those between H5 and H6 protons of a cytidine (distance, 2.46 Å) were observed (data not shown). This confirms that the resonances indicated by asterisks are those of the H8 protons of guanosines in the syn conformation. Judging from the one-dimensional spectrum (Figure 1c (upper)), most (~85%) of $r(CGCGCG)_2$ takes on the Z form under these conditions, consistent with the previous report.[19] It was shown in the same way that the resonances indicated by asterisks in Figure 1d (upper) are those of the H8 protons of guanosines in the syn conformation and that most (~80%) of $d(CGCGCG)_2$ takes on the Z form under these conditions.

The lower traces in Figure 1a–d are the photo-CIDNP difference spectra of the A, B, and Z form oligonucleotides, respectively. The CIDNP signals for H8 protons of guanines are clearly observable for the A and B form oligonucleotides, while those are very weak for the Z form oligonucleotides.

Prior to these experiments, the effect of $NaClO_4$ at high concentration on the CIDNP signal of 5′GMP was examined. The intensity of the CIDNP signal observed for the H8 proton of 5′GMP in saturated $NaClO_4$ was the same as that in 0.15 M NaCl (data not shown).

The Effect of Binding of Chromomycin on the CIDNP Signal of $d(GGGGCCCC)_2$. We have studied the structure of d-$(GGGGCCCC)_2$ by NMR and concluded that it takes on the B form.[9] The chemical shift temperature profile of base protons shows that the melting temperature of $d(GGGGCCCC)_2$ is over 60 °C and that the amount of melted single strands is completely negligible below 45 °C (data not shown).

We have studied the structure of the chromomycin–d-$(GGGGCCCC)_2$ complex, too.[9] On the basis of identified intermolecular NOEs (27 NOEs), the structure of the complex has been constructed. The main feature of the complex is that chromomycin binds as a dimer to the minor groove of d-$(GGGGCCCC)_2$, which is consistent with previous works performed with different DNA sequences.[21,22]

Figure 2 shows the effect of binding of chromomycin on the CIDNP signals. The assignment of base protons indicated was performed by us,[9] which was carried out under the same conditions (pH and concentrations of salt, the oligonucleotide, and chromomycin) as used in the present CIDNP study. The H8 protons of the central two guanosines, G3 and G4, in drug-free d-

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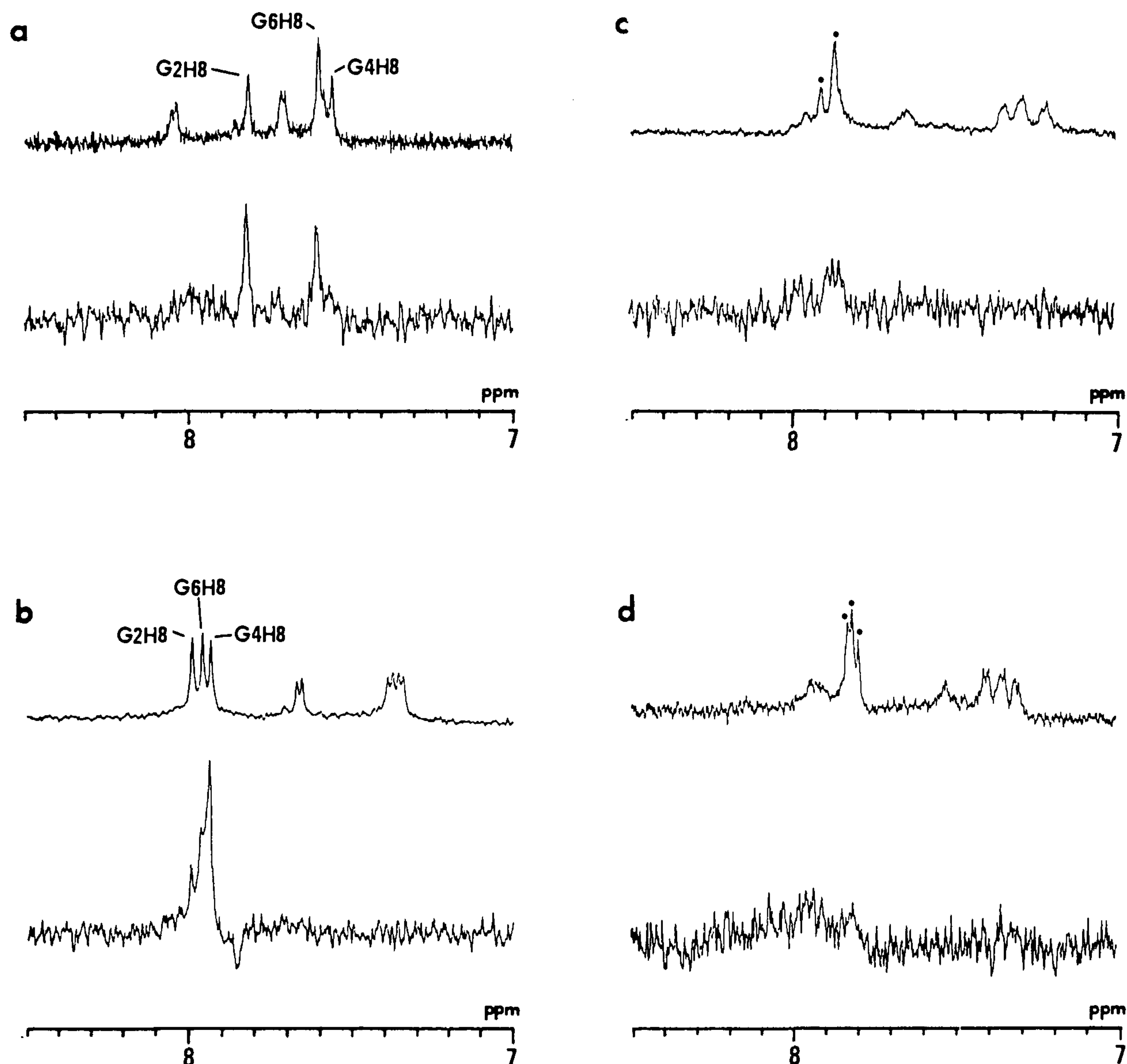

Figure 1. One-dimensional normal (upper) and photo-CIDNP difference (lower) spectra of (a) $r(CGCGCG)_2$ in 0.15 M NaCl (A form), (b) $d(CGCGCG)_2$ in 0.15 M NaCl (B form), (c) $r(CGCGCG)_2$ in saturated $NaClO_4$ (Z form), and (d) $d(CGCGCG)_2$ in saturated $NaClO_4$ (Z form) at 15 °C. Asterisks indicate the guanosine H8 resonances in the syn conformation (see text). The negative peak in the lower spectrum of (b) is due to flavin.

$(GGGGCCCC)_2$, their resonances being overlapping each other, give a clear CIDNP signal (Figure 2a). However, when d-$(GGGGCCCC)_2$ is bound with chromomycin, the CIDNP signal disappears (Figure 2b).

Prior to this experiment, the effect of the addition of chromomycin on the CIDNP signal of 5'GMP was examined. The intensity of the CIDNP signal observed for the H8 proton of 5'GMP was not affected at all by the addition of chromomycin under the same conditions as used for the experiment in Figure 2b (data not shown).

Selective Observation of the CIDNP Signal of an Unusual Cutting Site. The assignment of proton resonances of BL-1 and BL-2 was performed in the same way as in our previous works[23] by means of conventional 2D NMR methods, NOESY[24] and DQF-COSY[25] (Sakaguchi et al., unpublished result). A CIDNP signal was observed for the H8 proton of the terminal guanosine residue, G12, in the case of BL-1 (Figure 3a). In the case of BL-2, on the other hand, CIDNP signals were observed for the H8 protons of G4 and G3 residues, which are located just at the unusual cutting site, while the CIDNP signal was very weak for the terminal residue, G1 (Figure 3b).

Discussion

The CIDNP Signals of Double-Stranded Nucleic Acids. It has been believed that double-stranded nucleic acids do not give CIDNP signals, i.e., that only single-stranded nucleic acids give signals.[5] We, however, have observed clear CIDNP signals of double-stranded oligonucleotides (Figure 1). We carefully examined for possible traces of single-stranded oligonucleotides under the conditions with which the CIDNP experiments were carried out. In particular, for both $r(CGCGCG)_2$ in 0.15 M NaCl (A form) and $d(CGCGCG)_2$ in 0.15 M NaCl (B form), the published data[11,12] indicate that the melting temperature is over 50 °C and that the amount of single-stranded oligonucleotides is completely

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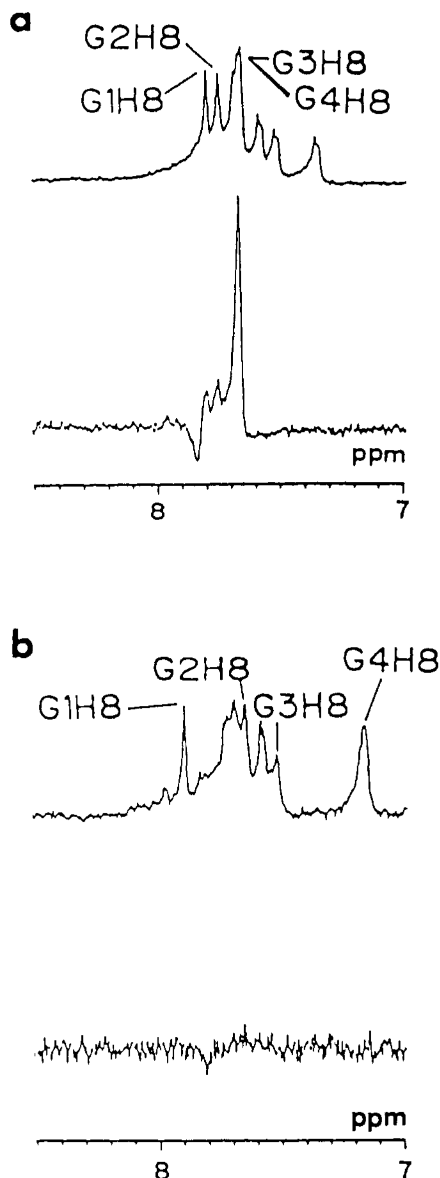

Figure 2. One-dimensional normal (upper) and photo-CIDNP difference (lower) spectra of (a) d$(GGGGCCCC)_2$ and (b) the chromomycin-d-$(GGGGCCCC)_2$ complex (two chromomycin molecules per duplex) in 0.15 M NaCl at 30 °C. The negative peaks in the lower spectra of (a) and (b) are due to flavin.

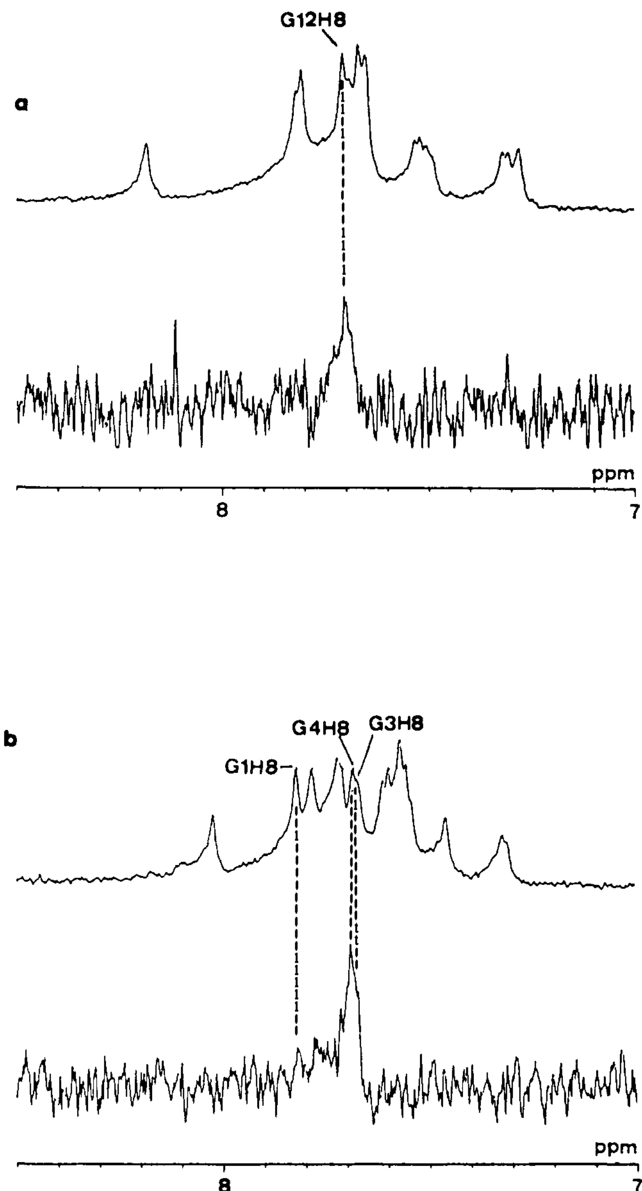

Figure 3. One-dimensional normal (upper) and photo-CIDNP difference (lower) spectra of (a) d$(CCCCAGCTGGGG)_2$ (BL-1) and (b) d-$(GGGGAGCTCCCC)_2$ (BL-2) in 0.15 M NaCl at 30 °C.

negligible below 20 °C, even with a low concentration of the oligonucleotides in comparison with that for the CIDNP experiments. The nonexistence of single-stranded oligonucleotides was further confirmed by the chemical shift–temperature profile of base protons obtained under the same conditions as those for the CIDNP experiments. Therefore, it is concluded definitely that the CIDNP signals in Figure 1 are due to the double-stranded oligonucleotides. Additionally, the fact that the most intense CIDNP signal is observed for the inner residue rather than the residue at the end, G6, of each oligonucleotide excludes the possibility that the CIDNP signals are just due to fraying of a duplex at the end (Figure 1a,b).

The observation of CIDNP signals for drug-free d-$(GGGGCCCC)_2$ confirms our claim that double-stranded nucleic acids give CIDNP signals, because the melting temperature of this oligonucleotide is even higher, over 60 °C, and the amount of melted single strands is completely negligible below 45 °C. An intense CIDNP signal is observed for the central two residues, G3 and G4, while those for G1 and G2 residues are much weaker (Figure 2a). This result once again excludes the possibility that the observation of the CIDNP signals is due to fraying at the end of the oligonucleotide duplex.

There is another possibility that the CIDNP signal comes from single-stranded segments of either slipped structure or hairpin structure in equilibrium with the double-stranded structure. This is, however, very unlikely, although it is difficult to deny it completely. First of all, the result on BL-2, that is, G3 and G4 give CIDNP signals but G6 does not, cannot be explained at all in this context. Secondly, any additional minor peak corresponding to those structures was not observed in 1D and 2D NMR spectra of studied oligonucleotides. Thirdly, the hairpin structure of a self-complementary oligonucleotide where guanine and cytosine residues form loop has not been found. Fourthly, hairpin structure is stable under much lower salt conditions than those used in this study.

Attempts to observe the CIDNP signals of double-stranded nucleic acids by previous workers were not successful for d-$(CGCG)_2$[8] and r$(AGCU)_2$.[5] A possible reason for the failure would be the use of riboflavin,[5] which has a bulkier side chain than flavin I used in our study and thus might be unable to gain access to the base in a duplex. The CIDNP signals of nucleic acids are weak in comparison with those of proteins, which might be the cause of the difficulty in detection due to the sensitivity of the NMR instruments. In the case of r$(AGCU)_2$, the decrease of the CIDNP signal generally observed at low temperature, even

for a mononucleoside, which is probably due to changes in T_1, the diffusion rate, and so on, as suggested previously,[5] might partly be responsible for the difficulty in observing the CIDNP signal of the duplex.

Interpretation of Selective Observation of the CIDNP Signal. In the case of drug-free d(GGGGCCCC)$_2$, an intense CIDNP signal was observed exclusively from the central two residues, G3 and G4. Similarly, an intense CIDNP signal was observed exclusively for the G12 residue of BL-1 and for the G3 and G4 residues of BL-2, respectively. One probable interpretation of the results is that those residues which exhibit the intense CIDNP signal would have higher affinity for flavin than the other residues. As far as three oligonucleotides mentioned above are concerned, there is one point common to those residues. That is, they are all located at the third or fourth position of the stretch consisting of four consecutive guanine residues. Some structural features responsible for high affinity for flavin might exist in this region. In the case of the stretch consisting of consecutive adenine residues, compression of the minor groove is observed particularly near the end of the stretch.[23,26] Therefore it is not unreasonable to expect some kind of particular structural features there.

It should be noted that unusual cutting of BL-2 by bleomycin occurs just at the same site where the CIDNP signal was observed selectively. Some structural features responsible for selective observation of the CIDNP signal would be related to unusual cutting by bleomycin.

The CIDNP signals are very weak for the Z form oligonucleotides (Figure 1). As the intensity of the CIDNP signal of 5′GMP is not affected by a high $NaClO_4$ concentration, this result might be caused by the structural features of the Z form which is responsible for low affinity for flavin.

Detection of Drug–DNA Interaction by the Photo-CIDNP Technique. As shown in Figure 2, when d(GGGGCCCC)$_2$ is bound with chromomycin, its CIDNP signals disappear, while the CIDNP signal of 5′GMP is not affected by the addition of chromomycin. Taking into account the elucidated structure of the chromomycin–d(GGGGCCCC)$_2$ complex,[9] the most likely explanation for the disappearance of the CIDNP signals on formation of the complex would be that chromomycin bound to the minor groove of d(GGGGCCCC)$_2$ interferes with the access of a flavin molecule to the base through direct blocking and/or inducing structural change of the oligonucleotide and thus prevents the photoreaction between a flavin molecule and the base indispensable for the appearance of the CIDNP signal. Thus it is suggested that the CIDNP signals of nucleic acids could be used to detect the interaction with drugs.

Conclusion

We have observed the photo-CIDNP signals of double-stranded nucleic acids for the first time. The technique can provide information on the local deviation of the nucleic acid structure, which may cause preferential binding of a flavin dye. This technique can also be used to detect the interaction between nucleic acids and drugs, as shown in the case of the chromomycin–d(GGGGCCCC)$_2$ complex. Although, until now, the photo-CIDNP technique has been applied almost exclusively to proteins, the results of the studies reported here show the possibility of the application of this technique to structural studies of nucleic acids.

Acknowledgment. This research was supported by a Grant-in-Aid for Special Project Research from the Ministry of Education, Science and Culture of Japan (No. 02263103). M.K. was supported partly by grants from the Japan Society for the Promotion of Science for Japanese Junior Scientists, the CIBA-GEIGY Foundation for the Promotion of Science, Takarazuka, Japan, and the Human Frontier Science Program for Long-Term Fellowships.

Registry No. BL-1, 136238-00-1; BL-2, 134649-59-5; r(CGCGCG)$_2$, 89435-90-5; d(CGCGCG)$_2$, 58927-26-7; d(GGGGCCCC)$_2$, 69374-98-7; d(GGGGCCCC)$_2$·chromomycin, 134846-92-7.

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