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In situ observation of DNA hybridization and denaturation by surface infrared spectroscopy

Ko-Ichiro Miyamoto, Ken-Ichi Ishibashi, Ryo-Taro Yamaguchi, Yasuo Kimura, Hisao Ishii, and Michio Niwano

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We have investigated in situ the hybridization and denaturation of DNA in aqueous solution using infrared absorption spectroscopy (IRAS) in the multiple internal reflection (MIR) geometry. We demonstrate that conformational changes of DNA strands due to hybridization (binding of two complementary single-stranded DNAs) and denaturation (separation of double helix at elevated temperatures) are reflected in the infrared absorption spectra in the frequency region where vibrational modes of the bases of DNA appear. Comparison with results of ab initio cluster calculation shows that hybridization produces the specific C═O carbonyl stretching vibration modes in the hydrogen-bonded base pairs. The ratio of absorbance of the C═O stretching peak at 1690 cm⁻¹ to the absorbance at 1660 cm⁻¹ provides a definitive metric for determining DNA hybridization. We also reveal that the C═O stretching vibration modes of the bases of a single strand is strongly influenced by the surrounding water molecules that may interact with the C═O groups of the bases. The present results suggest that MIR-IRAS is applicable to label-free, high-sensitive biosensors that provide insight about the gene expression and a variety of biological interactions such as DNA-protein interactions. © 2006 American Institute of Physics.

I. INTRODUCTION

In the field of genomics, the focus is now shifting to the gene expression, because the gene expression is closely related to some common diseases. Elaborate studies have so far been carried out about the gene expression related with some diseases: asthma¹,² and rheumatoid arthritis.³,⁴ To elucidate the details of the gene expression, it is important to analyze and to understand structural and functional relationships of biomolecules such as DNA, RNA, and proteins, in various clinical states. The conventional method of analyzing biological functions of biomolecules or cells is fluorescence microscopy in conjugation with labeling of biomolecules with various kinds of chromophores.⁵-⁷ Although this method has quite high sensitivity of detection of biomaterials, fluorescence labeling has some disadvantages: long-term sample preparation, cell toxicity, influence of chromophores on the binding nature of biomolecules, and so on. Infrared absorption spectroscopy (IRAS) provides an alternative method for the analysis of biological functions of biomedical samples and biomolecules.⁸-¹⁰ The advantage of IRAS over other methods is that IRAS is a nondestructive, noncontact photonic technique that provides a rapid measure of sample chemistry; it is capable to reveal detailed conformational changes of biomolecules such as DNA and proteins through infrared absorption spectral patterns. Furthermore, fluorescence labeling or radioactive labeling is not necessary for distinguishing reacted and unreacted chemical species. However, there are very few reports on the application of IRAS to biosensors.¹¹ We have previously proposed a method of in situ analyzing the bonding conformation of DNA in aqueous solution by IRAS in the multiple internal reflection (MIR) geometry.¹² MIR-IRAS is intrinsically sensitive to surface vibrations,¹³,¹⁴ and consequently is suitable for biosensors in which only surface phenomena are involved. We have revealed that DNA hybridization can be determined by measuring IRAS spectra of DNA.¹²

In this study, we have investigated in more detail how hybridization as well as the so-called denaturation induces IRAS spectral modifications for DNA in aqueous solution. To do this, we have selected the chemical system of complementary, single-stranded DNAs (ss-DNAs). The IRAS spectral changes caused by DNA denaturation have been already reported for a simple base pair of dA₁₂-dT₁₂ (Ref. 15) and for a more complicated, calf thymus DNA.¹⁶ In this study, we employ 30-mers oligonucleotides as the appropriate template for testing the possible application of MIR-IRAS to biosensors for detecting DNA hybridization. We clearly observe IRAS spectral modifications upon hybridization of complementary pairs of oligonucleotides (ss-DNAs), and also in situ observe spectral changes caused by denaturation of double-stranded DNAs (ds-DNAs) at elevated temperatures. From a comparison with results of ab initio cluster calculations, we determine that hybridization produces some specific C═O carbonyl stretching vibration modes in the hydrogen-bonded base pairs. We also demonstrate that the C═O stretching vibration modes of the bases of a single strand is strongly influenced by the surrounding water molecules that may be bound to C═O carbonyl groups of the bases through hydrogen bonding.
II. EXPERIMENT AND ANALYSIS

A. MIR-IRAS measurements

Figure 1 illustrates the electrochemical cell we used in this study for MIR-IRAS measurements. The volume of the sample solution was 100–150 μl. A Si prism was 0.5 × 10 × 30 mm³ with 45° bevels on each of the short edges, and contacts with the sample solution. Gold thin films were deposited onto the backside of the prism. The gold films were used as the electrodes for resistive heating of the Si prism. Resistive heating of the Si prism elevated the temperature of the sample solution. A thermocouple was attached to the electrochemical cell to monitor the solution temperature. The temperature was varied in the range of 30–60 °C by applying different voltages between the pair of electrodes on the backside of the sample. Figure 2 shows a typical evolution of the solution temperature while resistively heating. When the solution temperature was stabilized, we started to collect IRAS spectra, and a given solution temperature was maintained while IRAS spectra were collected.

An infrared light beam from an interferometer (BOMEM MB-100) was focused at normal incidence onto one of the two bevels of the Si prism, and penetrated through the Si prism, internally reflecting about 60 times. The light that exited the Si prism through the other bevel was focused onto a liquid-nitrogen cooled mercury-cadmium-telluride (MCT) detector.

B. DNA samples

We used four types of 30-mers oligonucleotides (ss-DNAs) that were synthesized and subsequently purified by high-pressure liquid chromatography (HPLC) by Nihon Gene Research Laboratories Inc., Sendai, Japan. We denote the oligonucleotides as L, L’, R, and R’. The base sequence of oligonucleotide L was 5’-GGAG ACTG TTAT CCGC TCAC AATT CCAC AC-3’. R was complementary to L. R’ had the same base composition as R, but was not complementary to L; R’ had a base sequence of 5’-TAGC TGTA CTGG TATG CAAG ACGC TGTT AG-3’. L’ had a base sequence of 5’-GGAG ACTG TTAT TTTC TCAC AATT CCAC AC-3’; it had a four-based mismatch in the center portion of the strand to R. These single-stranded oligonucleotides were solved in a solution of NaCl (typically 14.3 mM) in heavy water, D₂O. The reason why we used D₂O instead of H₂O as the solvent is that H₂O has the strong scissoring peak around 1640 cm⁻¹ where the bases of DNA have specific vibration modes (C==O, C–N stretching, and also NH₂ scissoring modes) that are quite sensitive to base pairing, that is, hydrogen bonding. On the other hand, D₂O has no significant vibration modes at these wavelength regions; it has scissoring mode at 1230 cm⁻¹. The concentration of oligonucleotides in D₂O solution was typically 75–100 μM.

C. Ab initio cluster calculation

In order to determine the origins of the IRAS spectral modifications caused by DNA hybridization and denaturation, we have carried out a detailed theoretical analysis of the vibration frequencies of the base part of DNA. To simulate the DNA molecule, we employ a nucleotide that consists of a base and a five-carbon sugar and one phosphate group. In electronic structure calculations, we adopt the well-established hybrid density-functional theory (DFT) (B3LYP) with the basis set of the polarized double-zeta Gaussian-type orbitals (6-31G**). Based on Becke’s method, the electron many-body energy functional is a mixture of the exact exchange energy and of the exchange correlation energy in the conventional DFT (the local density approximation and generalized gradient approximation). We first optimized the geometries of the cluster, and then obtained the vibration frequencies of vibrational modes of the base. All the calculations are performed using the GAUSSIAN 98 program, Gaussian Inc. We have introduced the scaling factor of 0.9613 for the 6-31G** basis set; this value is widely used in evaluating vibration frequencies.

III. RESULTS AND DISCUSSION

A. DNA hybridization

First, we consider the case in which two single-stranded DNAs (oligonucleotides) are not complementary to each other. In Figs. 3(b) and 3(c) are shown typical IRAS spectra of the individual oligonucleotides L and R’, respectively. The
DNAs exhibited absorption peaks in the frequency region of 1500–1750 cm⁻¹, which are due to the vibration modes of the bases.¹⁵ As described below, the dominant peak located around 1660 cm⁻¹ originates from the C=O carbonyl stretching vibration modes of the bases of guanine (G), cytosine (C), and thymine (T). We see that spectral profiles are quite similar but reveal small differences. The similarity in spectral profile between L and R’ is mainly due to the overlap of different base contributions, because both nucleotides have all the kinds of bases with almost the same base composition. However, close inspection into the two spectra indicates that the main peak in the spectrum of L is positioned at a slightly higher frequency than that in the spectrum of R’. Note that the base composition of R’ is A:T:G:C = 7:8:10:5, while that of L is 8:7:5:10; R’ has twice more bases of guanine than L. As described below, the base guanine has the C=O stretching vibration mode located at a higher frequency than the base cytosine. We therefore suppose that the observed difference in the peak position is due to the difference in the base composition. This point will be discussed later again, in comparison with results of cluster calculations.

In Fig. 3(a), we plot a typical IRAS spectrum collected for a mixed solution of oligonucleotides L and R’ in D₂O. For comparison is shown a computed spectrum, indicated by a thin curve, (L)+(R’), in Fig. 3(a), that was obtained by simply summing the two individual spectra of oligonucleotides L and R’ with equal weight. In Fig. 3(a), the experimental spectrum of the mixed solution and the computed spectrum were normalized to the IR absorbance at 1664 cm⁻¹ where it is indicated by a dashed line in Fig. 3. No distinct difference can be identified between the experimental and computed spectra. This suggests that there was no specific interaction between L and R’; that is, hybridization did not occur.

Figure 4 shows the results we obtained for the two complementary oligonucleotides, L and R. In Fig. 4(a) is shown a typical IRAS spectrum collected for the DNA solution in which the two oligonucleotides were mixed up. As in Fig. 3(a), a thin curve indicates the computed sum of the two spectra of the individual nucleotides, shown in Figs. 4(b) and 4(c), and the experimental spectrum of the mixed solution and the computed spectrum were normalized to the absorbance at 1664 cm⁻¹. Comparison between the experimental and computed spectra clearly indicates that the experimental spectrum, indicated by (L+R), exhibits notable enhancement in absorbance at 1690, 1670, and 1640 cm⁻¹, as compared to the computed spectrum, (L)+(R). We interpret that the observed spectral difference indicates the hybridization between the two complementary oligonucleotides. It has previously been reported¹⁵ that spectral features located around 1690 cm⁻¹ can be attributed to the C=O stretching modes of thymine and guanine residues, and those located around 1650 cm⁻¹ are due to the ring deformation modes of thymine residue. We therefore suppose that these vibrational modes may be affected by conformational changes due to DNA hybridization. This point will be in more detail discussed below, in comparison with results of ab initio cluster calculations.

B. DNA denaturation

We have in situ investigated how the IRAS spectral profile changes during denaturation of DNA in a NaCl solution. In the lower portion of Fig. 5 is shown the denaturation curves of the nucleotide pair of L-R that is a plot of UV absorbance at 260 nm of L-R against the sample temperature. It is well known that the UV absorbance at 260 nm of DNA strongly depends on its conformation; the 260 nm absorbance is enhanced when ds-DNAs are denatured into ss-DNAs, and on the other hand, it is reduced when two complementary ss-DNAs hybridize to form ds-DNAs.⁻¹⁹ This phenomenon therefore has been widely used to monitor DNA hybridization. We can see from Fig. 5 that the double helical structure of the L-R pair denatured between 50 and 60 °C. When the temperature was decreased, the double helical structure was recovered at temperatures below 50 °C. We plot in Fig. 6 a series of IRAS spectra of the solution containing L and R, recorded while the solution temperature was ramped up to 60 °C and subsequently was decreased to 30 °C. The figure attached to each curve indicates the temperature of the solution at which IRAS spectra have been measured. The bottom spectrum recorded at 30 °C corre-
sponds to the starting point of DNA denaturation; it exhibits the same spectral profile as in Fig. 4, indicating the double helical structure. When the solution temperature was raised up to 50 °C, the spectral profile did not change significantly, indicating the retention of the double helix structure up to this temperature. This is consistent with the results of UV absorption measurements shown in Fig. 5.

When the temperature was increased to 60 °C, the spectral profile changed; a distinct change is a decrease in the absorbance around 1690 cm⁻¹. The resulting spectral profile is quite similar to those observed for the single-stranded oligonucleotides, shown in Figs. 3 and 4. This indicates that DNA denaturation occurred at this temperature, and is consistent with the results of UV absorption measurements shown in Fig. 5.

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1690 cm$^{-1}$ to that at 1660 cm$^{-1}$, also exhibited the temperature dependence that is consistent with the UV absorbance. Thus, the present results show that we can in situ monitor DNA hybridization and denaturation by MIR-IRAS, and also that the absorbance at 1690 cm$^{-1}$ reflects the hybridization of DNA; that is, the ratio of absorbance of the C=O stretching peak at 1690 cm$^{-1}$ to the absorbance at 1660 cm$^{-1}$ provides a definitive metric for determining DNA hybridization, as the UV absorbance at 260 nm does.

C. Comparison with cluster calculation

The present experimental results clearly showed that DNA hybridization induced some IR spectral changes in the frequency region where the vibrational modes of the bases appear. This is not surprising because hydrogen bonding between complementary base pairs is involved in DNA hybridization. Figure 9 illustrates the hydrogen bonding between the A-T and G-C base pairs. We see that the C=O carbonyl stretching vibration modes may be affected by hydrogen bonding. We have investigated therefore how DNA hybridization affects vibrational modes of the bases by calculating the vibration frequencies based on the *ab initio* cluster calculation. The model systems of the base pairs we presented in Fig. 9 were employed in the calculation.

We plot in Fig. 10 frequencies and oscillator strengths of the vibrational modes that originate from the four individual nucleotides and the base pairs, A-T and G-C. To facilitate the comparison with the experimental spectra, we computed the spectra of the individual bases and the DNA pairs by convoluting the calculated oscillator strengths using a Gaussian function with a width of 20 cm$^{-1}$. The results are shown by broken curves in Fig. 10. The vibrational modes that show up in the frequency region we investigated here are mainly due to the C=O stretching vibrations and the purine or pyrimidine ring deformation modes. Brewer *et al.*$^{11}$ have previously derived similar spectra using cluster calculations based on the DFT. Their spectra are different in spectral shape from ours. The reason for the discrepancy would be as follows. One is the size of model clusters. The clusters that Brewer *et al.* utilized in their calculations are smaller than ours; they have considered only the base part of DNA, while we used a nucleotide that consists of the sugar and phosphate parts beside the base. Another reason would be the replacement of hydrogen atoms of the N–H bonds of the bases by deuterium. We have collected IR spectra of DNA in D$_2$O solution. It is
well known that the hydrogen atoms of the N–H bonds readily exchange with deuterium in aqueous solution of D₂O. We have therefore calculated the vibrational frequencies using the bases having N–D bonds.

In Fig. 11, we show the experimental spectra for 10-mer oligonucleotides of four bases (dA₁₀, dT₁₀, dG₁₀, and dC₁₀), compared with the calculated ones. As will be described below, all the calculated spectra have been shifted by 20 cm⁻¹ compared with the calculated ones. As will be described below, all the calculated spectra have been shifted by 20 cm⁻¹ to higher frequencies. We see from Fig. 11 that the calculation cannot explain the experiment, especially the peak positions of the C–O stretching vibration modes. The C–O stretching vibration peaks in the experimental spectra are positioned at lower frequencies than the calculated ones. We interpret that this discrepancy is due to the interaction of the C–O bonds with the surrounding water molecules; that is, the hydrogen bonding of C–O carbonyl groups with the D₂O molecule. As seen in Fig. 11, a 10-mer oligonucleotide of thymine, dT₁₀, displayed a three-peaked feature in its experimental IRAS spectrum. The ab initio cluster calculation predicted that this spectral feature can be attributed to the hydrogen bonding of the two C–O groups of the base thymine with the heavy water molecule. This is the case for the other oligonucleotides. This point will be discussed in more detail elsewhere.

As can be seen in Fig. 11, the cluster calculation predicted that the base guanine exhibits the C–O stretching vibration mode at a higher frequency than the cytosine. The experimental spectra of Fig. 11 also show that the main peak of the guanine nucleotide was positioned at a slightly higher frequency than that of the base cytosine one. As mentioned in the discussion about the results of Figs. 3 and 4, this would be the reason why the main peak in the spectrum of oligonucleotide R’ is positioned at a slightly higher frequency than that in the spectrum of L; R’ has twice more bases of guanine than L.

Figures 12(a) and 12(b) show the calculated spectra of the A-T and G-C pairs, respectively. For comparison are shown the calculated spectra obtained by addition of the spectra of the individual bases (thin curves). The latter spectra indicate the simulation of the separated base pairs. The hydrogen-bonded base pairs exhibit quite different spectral profiles from the corresponding separated base pairs, which suggest that hybridization causes significant spectral modifications in the IRAS spectra of the base pairs. It is apparent from Fig. 10 that the C–O stretching vibrations are greatly influenced by hydrogen bonding between the base pairs, while the pyrimidine or purine ring deformation modes are not affected by hydrogen bonding. We compare in Fig. 12(c) the calculated spectrum that is the sum of the spectra of Figs. 12(a) and 12(b), with the experimental spectra obtained for the L-R pair. As described above, oligonucleotides L and R, have the same number of A-T and G-C pairs, and therefore, the calculated spectrum has been obtained by addition of the calculated spectra of the A-T and G-C pairs with the same weight. In addition, the calculated spectrum was by 20 cm⁻¹ shifted to higher frequencies, so as to give a good fit to the experimental one; the calculated peak position of the purine ring deformation mode, which is not strongly affected by hybridization, was fitted to the experimental one, 1570 cm⁻¹, as is shown in Fig. 12. Overall agreement in spectral profile between the experiment and calculation can be identified in the spectra of Fig. 12(c). As described above, we observed that hybridization caused a notable enhancement in absorbance at 1690, 1670, and 1640 cm⁻¹. Arrows in Fig. 12 indicate these positions. We can see from a comparison of Figs. 10 and 12 that the observed spectral modifications are due to the conformational changes around the C–O bonds that are involved in hydrogen bonding between the base pairs. Here, it should be pointed out that the hydrogen-bonded base pair is not so much affected by the surrounding water molecules as the single-stranded DNA is. This is because the base pairs of the double-stranded DNA are surrounded by the two backbones and moreover almost all the C–O bonds are involved in hydrogen bonding, as is illustrated in Fig. 9.
IV. CONCLUSIONS

We have in situ investigated the hybridization and denaturation of DNA in aqueous solution using infrared absorption spectroscopy (IRAS) in the multiple internal reflection (MIR) geometry. We demonstrated that conformational changes of DNA strands due to hybridization and denaturation are reflected in the infrared absorption spectra of the base part of DNA. Comparison of IRAS data with results of ab initio cluster calculations showed that hybridization produces the specific C—O stretching vibration modes in the hydrogen-bonded base-pairs, and also that the C—O stretching vibration modes of the bases of a single strand may be strongly influenced by the surrounding water molecules that may interact with the C—O carbonyl groups. The present results suggest that MIR-IRAS is applicable to label-free, high-sensitive biosensors that provide us with detailed information about interactions between biomaterials. MIR-IRAS facilitates the monitoring of conformation changes of biomaterials such as proteins, lipids, and cells, besides DNA. It should be pointed out that no fluorescence tags and radioactive tags are necessary in our IRAS method. Investigations on the interactions between proteins and the functions of cells using the present method are now in progress.

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