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Hydration of single-stranded phosphodiester and phosphorothioate oligodeoxyribonucleotides

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ABSTRACT

Infrared spectroscopy was used to identify hydration-sensitive structural differences between single-stranded phosphorothioate (PS) and phosphodiester (PO) oligodeoxyribonucleotides. Spectra were recorded in the mid-infrared region, 500–1800 cm^{-1}, at relative humidities between 0 and 98%; the PS and PO spectra are substantially different. The hydration effects on spectral bands in these single-stranded oligodeoxyribonucleotides is markedly different from such behavior in double- and triple-stranded oligodeoxyribonucleotides. A strong absorption occurs at 656 cm^{-1} in the phosphorothioate sample which is completely absent from the PO spectra. Gravimetric measurements were carried out on one PS and one PO sample to monitor and confirm hydration. The calculated BET adsorption constants [Brunauer,S., Emmett,P.H. and Teller,E. (1938) *J. Am. Chem. Soc.*, 60, 309–319] are 1.2 and 1.4 water molecules per nucleotide in the first hydration layer of PS and PO respectively. While the gravimetric data indicate that the single-stranded oligodeoxyribonucleotides hydrate very similarly to duplex DNA, the mid-infrared conformational marker bands are strikingly different from those observed for duplex DNA. In particular, the ν_{as} of the phosphate group (PO_2) at 1222 cm^{-1} in the single-stranded PO spectra is independent of relative humidity.

INTRODUCTION

Solvent plays a critical role in stabilizing the functionally relevant conformation of biomolecules. Because physiological systems function by manipulating the water activity of biomolecular interactions, our comprehension of the orchestration of macromolecular form and function is intimately tied to an understanding of H_2O and its interactions with biomolecules at the molecular and sub-molecular level. Hydration has been characterized as a critical and biologically relevant stability parameter, a standard example of which is the effect of the spine of hydration of poly(dA)·poly(dT) (1). It has been reported that the water molecules that are directly bound enthalpically stabilize the structure of oligodeoxyribonucleotide duplexes (2).

Perhaps the most fundamental experiment involving water molecules and DNA is to determine how many H_2O molecules there are in the neighborhood of a nucleotide; one method of achieving this objective is by weighing the amount of water adsorbed to the DNA. The BET equation describes the adsorption of a gas on a substrate, and is based on the amount of condensed gas contained in the first adsorption layer and the difference between the binding energies of the first and subsequent layers (3). Experimentally, the number of water molecules bound to an oligodeoxyribonucleotide can be determined by gravimetric analysis measuring the weight of water adsorbed as a function of relative humidity and then fitting the data to the BET adsorption equation which will be presented in the Materials and Methods section.

A considerable part of present research on oligodeoxyribonucleotides is motivated by their demonstrated ability to selectively inhibit gene expression. Both antisense DNA–RNA hybrid duplex formation and oligodeoxyribonucleotide directed triple helix formation are being investigated as mechanisms of action (4–9). The phosphorothioate internucleotide linkage in synthetic oligodeoxyribonucleotides is the most extensively studied modification to the 'normal' nucleic acid structure for two primary reasons. First, PS oligodeoxyribonucleotides have demonstrated enhanced resistance to nucleolytic degradation when compared with the unmodified phosphodiester. Secondly, the sulfur containing oligodeoxyribonucleotides appear to be transported comparatively rapidly across cell membranes, a favorable property from a pharmacokinetic standpoint. Despite the intense interest in these molecules, little is known of their structure and dynamics at the atomic level. Falk *et al.* have reported the gravimetric results for unmodified duplex DNA of essentially random sequences but to the best of our knowledge, there are no published hydration studies of single-stranded oligodeoxyribonucleotides (10). In this paper, we report a gravimetric and a Fourier Transform infrared spectroscopic comparison of single-stranded phosphorothioate and phosphodiester oligodeoxyribonucleotides. The long-range goal of these hydration and backbone vibration studies is to develop a biochemically relevant atomic-level description of factors affecting the hybridization stability of oligodeoxyribonucleotides (11). Such an understanding is vital in

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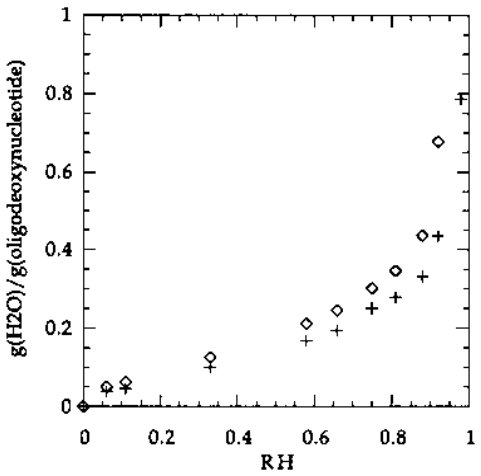

Figure 1. Gravimetric hydration data of the *GCC GAG GTC CAT GTC GTA* CGC oligodeoxyribonucleotides with phosphodiester linkages (◇) and with phosphorothioate linkages (+).

order to more rationally design oligodeoxyribonucleotides for pharmaceutical applications.

MATERIALS AND METHODS

Sample preparation

Oligodeoxyribonucleotides were prepared via solid-phase synthesis on an ABI 380B synthesizer (Applied Biosystems, Foster City, USA) with commercial phosphoramidates (Millipore, Boston, MA, USA). During synthesis the P(III) phosphite bond was oxidized to the P(V), phosphate triester with either aqueous I_2 or Beaucage reagent to generate the P=O or P=S bonds respectively. Following deprotection and HPLC purification sample purity was assessed using capillary gel electrophoresis. All samples contained ≥95% full length product. Phosphorothioate compounds contained <8% single PO substitutions as determined by ^{31}P NMR spectroscopy. Two sequences were prepared: a 20mer TGC ATC CCC CAG GCC ACC AT, as a diester [Isis 8651] and phosphorothioate [Isis 3082] linkages and a 21mer GCC GAG GTC CAT GTC GTA CGC (PO, Isis 1049; and PS, Isis 1082 respectively).

The technique for varying hydration to a desired number of water molecules per nucleotide is well established (12). Constant humidity levels were established and regulated within sealed chambers by the continual exchange of water molecules between the air and a saturated salt solution at 25°C, or with Drierite at 0% RH. The relative humidity (RH) levels used in the experiments and their associated salt solutions are as follows: 33%, $MgCl_2$; 58%, NaBr; 66%, $NaNO_2$; 75%, NaCl; 81%, $(NH_4)_2SO_4$; 88%, K_2CrO_4; 92%, $Na_2C_4H_40_6$; 98%, $CaSO_4$. A capacitance based RH meter (Omega Scientific model RH-200) was used to experimentally confirm relative humidity and temperature within the desiccators. All samples were allowed to equilibrate for ≥24 h in environments of known and increasing RH.

Gravimetric measurements

Films of Isis 1049 and Isis 1082 were dried for 24 h in an evacuated glass desiccator containing phosphorous pentoxide (P_2O_5) and then weighed in polyethylene tubes using a Cahn microbalance (±0.02 mg). Samples were then hydrated as described previously (10). (0% RH does not imply the complete absence of structural water molecules associated with the DNA. In calf thymus DNA there are approximately six water molecules per base pair at 0% RH (13). These water molecules are considered to be part of the dry sample; the adsorption curve represents hydration beyond this point.) In order to obtain accurate BET constants, the samples were measured at two additional relative humidities: 6% using NaOH and 11% using LiCl. Relative humidity was determined to be accurate within 2% RH, and the samples were weighed after 24 h to ensure that equilibrium was reached. This hydration and weighing procedure was also applied to empty tubes in order to account for the adsorption of water on the polyethylene tubes.

Our method of analyzing the gravimetric data is based on the BET equation. This equation was given a statistical mechanical foundation by Hill (14), and in Hill's notation it is given as

$$A = \frac{BCx}{(1 - x + Cx)(1 - x)} \quad \mathbf{1}$$

where, for our purposes, A is the mass of water per gram of phosphorothioate adsorbed at relative humidity x, B is the amount of water contained in the first hydration layer, and C is given by

$$C = De^{(E_1 - E_L)/RT} \quad \mathbf{2}$$

E_1 is the binding energy of the first layer, and E_L is the energy of subsequent layers, which can be assumed to be equal to the binding energy of liquid water. D is a constant which involves partition functions of water in the first and subsequent layers (15). Brunauer, Emmett and Teller suggested that D be set equal to 1, but this causes the calculated difference in binding energies to be low by as much as 1 kcal/mol (10). Since the BET equation is not very sensitive to changes in C, we cannot extract any meaningful information about C.

Spectroscopy

The complete set of RH measurements on both PS and PO oligodeoxyribonucleotides was repeated twice with highly similar results. Relative humidities were maintained under vacuum in a custom-designed airtight mid-infrared-transparent sample cell by a saturated salt solution or desiccating agent within the cell. Samples of the oligodeoxyribonucleotides were mounted for spectroscopy by applying them to one of the sample cell's ZnSe windows as an aqueous solution, then desiccating them to form a film. Infrared spectroscopy was performed at room temperature on a Bruker 113v Fourier transform infrared spectrometer. References were taken at each RH to compensate for the effect of water vapor in the sample chamber. 300 scans of the sample and reference were taken. The active element of the detector was DTGS at room temperature. The spectra we present are unmodified; no Fourier deconvolutions or subtractions have been performed.

RESULTS

The gravimetric adsorption curves for the phosphorothioate samples are plotted in Figure 1. The spectra of the PO and PS oligodeoxyribonucleotide samples for nine relative humidities are presented in Figures 2 and 3, respectively. Appropriate constants have been added to each of the spectra to allow for convenient comparison between adjacent hydration states. The

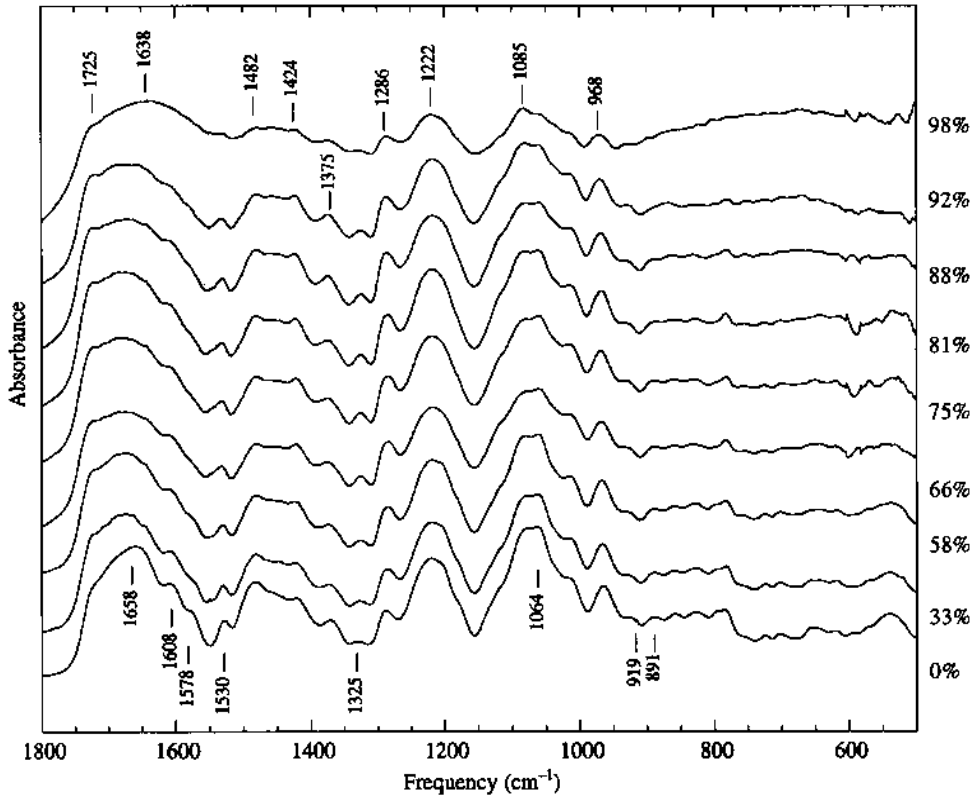

Figure 2. Spectra of the hydration series of the *TGC ATC CCC CAG GCC ACC AT* oligonucleotide with phosphodiester linkages.

absorption peaks are labeled with their peak frequencies for purposes of discussion.

DISCUSSION

Gravimetric analysis: the BET theory

The curves presented in Figure 1 are very similar in shape to those previously obtained for unmodified duplex DNA of essentially random sequence (10). To the best of our knowledge there are no published hydration studies of single-stranded oligodeoxyribonucleotides with which to compare our results.

The data shown in Figure 1 have been fit with the BET curve using KaleidaGraph 3.0.5, yielding χ^2 values of 11.5 for Isis 1049 and 12.2 for Isis 1082. Up to 81% RH, the data fit the BET equation well. The best values of the parameters were found to be B = 0.084 g H_2O/g phosphorothioate and C = 20 for the Isis 1049 sample, and B = 0.066 g H_2O/g phosphorothioate and C = 20 for the Isis 1082 sample. The uncertainties in the values of C make them uninterpretable. The values of B correspond to 1.4 molecules of water per nucleotide for Isis 1049 and 1.2 molecules of water per nucleotide for Isis 1082. In view of the fit obtained up to 81% RH and the previous application of the BET equation to DNA (10), it seems reasonable to conclude that the BET model applies to phosphorothioate as well as unmodified DNA. That is, water is adsorbed in layers around the primary adsorption sites, which are generally taken to be the phosphate groups in duplex DNA. The PO backbone adsorbs slightly, but significantly more water than the PS backbone. As in unmodified double-stranded DNA (10), we find that both sequences exhibit a negative deviation from the BET equation above 81% RH, i.e. the data lie below the BET curve beyond this point. This has been attributed to the onset of swelling in the fibers, indicating that the space between the fibers had been filled (10). Since we are working with single-stranded oligodeoxyribonucleotides rather than with long fibers of double-stranded DNA, however, the same argument may not hold. That this deviation is present in samples of widely differing mesoscopic structure may indicate that it is due to the mechanism of hydration at the molecular level. Further research is required to substantiate this suggestion.

Gravimetric measurements were made on a sample of the spectroscopic sequence (Isis 3082). They are not reported here because the sample used was less massive, therefore yielding less accurate BET constants, and because there are no significant differences between the results obtained for the two sequences.

Spectroscopic analysis

Two methods of comparison are considered here: (i) the spectral differences between the individual sample hydration states, and (ii) the spectral differences between phosphorothioate and phosphodiester samples at corresponding RHs. The detailed analysis proceeds by considering four spectral regions beginning with the lowest: furanose (500–1000 cm^{-1}), phosphate backbone (1000–1250 cm^{-1}), glycosidic (1250–1500 cm^{-1}) and base stacking (1500–1800 cm^{-1}). Before proceeding with a comprehensive explanation of the bands the highlights are presented:

(i) In Figure 2 the 1222 cm^{-1} ν_{as} PO_2 absorption of single-stranded PO oligonucleotides does not change its frequency over the entire range of RH from 0 to 98% despite demonstrated H_2O uptake (Fig. 1). This observation is in sharp contrast with the ν_{as} PO_2 band RH behavior in double helical DNA (16,17).

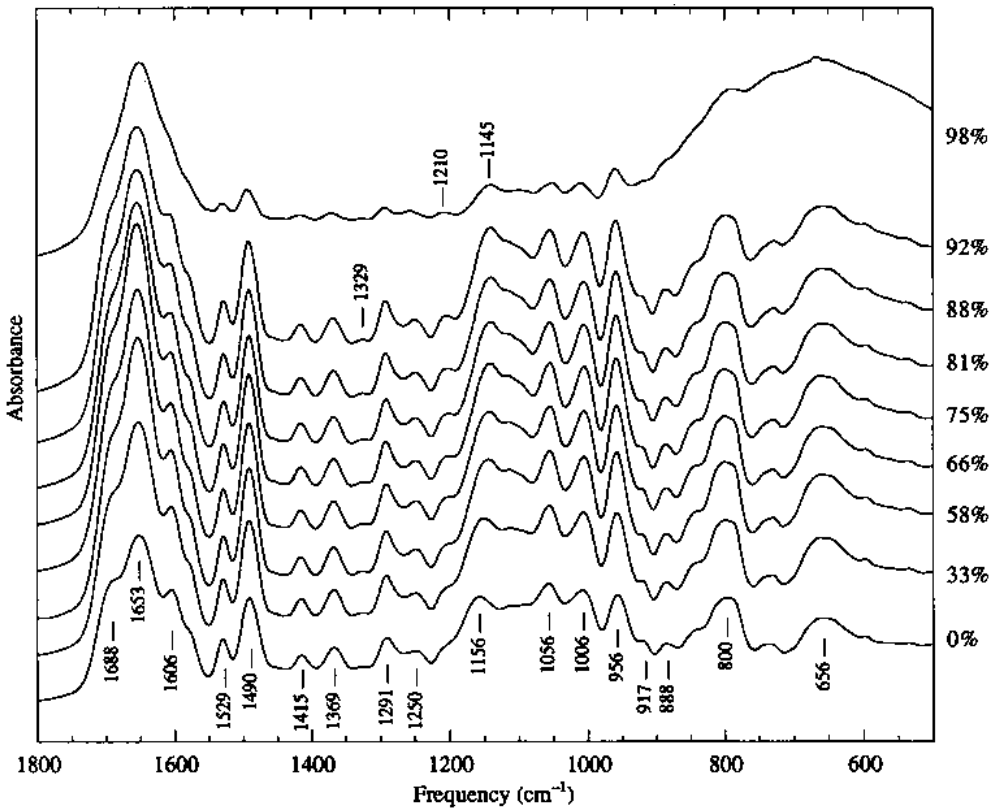

Figure 3. Spectra of the hydration series of the *TGC ATC CCC CAG GCC ACC AT* oligonucleotide with phosphorothioate linkages. The absorption of the 1653 cm^{-1} mode at 0% RH is 0.60 absorption unit. The x-axis for the 0% relative humidity spectrum represents 0.00 absorption unit.

(ii) In Figure 3 the 1156 cm^{-1} band of PS oligodeoxyribonucleotide decreases nearly 10 cm^{-1} in frequency with hydration increase as above.

(iii) PS and PO oligodeoxyribonucleotide spectra are markedly different at all RHs.

(iv) The strong 656 cm^{-1} band in PS spectra essentially does not appear in PO spectra.

Furanose frequency region

For all frequency regions the spectroscopic analysis will proceed in the following order: PO oligodeoxyribonucleotide spectra from 0 to 98% RH, similarly for PS oligodeoxyribonucleotide, concluding with a comparison of PO and PS oligodeoxyribonucleotide spectra beginning at low RHs.

The PO spectra show an increase in water absorption between ~500 and 800 cm^{-1} with respect to increase in RH, which confirms, in a qualitative manner, the quantitative results of Figure 1.

The spectra at all RHs between 500 and 1000 cm^{-1} do not display the traditional marker bands—such as 860 cm^{-1}—in the same way that double helical DNA does. The 0% RH spectrum appears similar to calf thymus spectra at 0% RH, thereby adding novel support to the destabilized double helix model for 0% X-ray results.

PS oligodeoxyribonucleotide spectra in the furanose region are much more structured than their PO analog. The broad and very strong band at 656 cm^{-1} exists at all RHs except at 98% RH where water absorption obscures its existence. The non-Lorentzian PS band nominally at 800 cm^{-1} similarly distinguishes itself from the corresponding region of PO spectra because of relatively great oscillator strength. Furthermore, this band is completely hydration insensitive, whereas a double helical band of marked hydration sensitivity exists at 806 cm^{-1} for low RHs and increases frequency by nearly 30 cm^{-1} at 92% RH has been reported (18). In adenine double helical polynucleotides the 800 cm^{-1} mode is of subordinate strength and in those spectra the absence of the 862 cm^{-1} band has been reported (16).

Comparison of the furanose region of PO and PS oligodeoxyribonucleotides indicates noteworthy differences which may lead to a deeper understanding of the precise nature of the P–S force constant. Further work with model compounds is required to unambiguously assign the 656 cm^{-1} band in the PS oligodeoxyribonucleotide to the PS stretch, however, its absence in PO oligodeoxyribonucleotide spectra supports this contention. A more subtle contrast in frequency between the PS and PO spectra is the 12 cm^{-1} difference in bands at center frequencies of 956 and 968 cm^{-1}, respectively. The integrated intensities of these two modes, however, are markedly different—the PS mode at 956 cm cm^{-1} being one of the hallmarks of the PS single-stranded spectra because of unusually high intensity relative to the principal bands of the phosphate backbone region. The oscillator strengths of the 956 and 968 bands were obtained by computations on the Bruker Aspect 3000 computer; oscillator fits (6 oscillators) were performed after baseline correction on the frequency interval 850–990 cm^{-1}. The 957.3 and 964.8 cm^{-1} center frequency Lorentzians integrated to 8.5 and 2.0 absorbance·cm^{-1} in PS and PO, respectively. The eigenvector assignment of these modes is most likely a furanose stretch and more investigation is needed to

explain the role of the sulphur substitution in increasing the oscillator strength of this band.

Phosphate backbone frequency region

This frequency region contains the canonical double helical absorptions due to the symmetric and antisymmetric stretches of the PO_2 group which intuitively would be most profoundly affected by the sulfur substitution which yields phosphorothioate single-stranded oligodeoxyribonucleotides.

In the PO spectra the 1222 cm^{-1} band is completely hydration insensitive, a fact which motivated several repetitions of this lengthy set of measurements as well as the gravimetric measurements. The other canonical PO_2 band, the symmetric stretch, is present at 1065 cm^{-1} and its frequency is hydration independent as is the case in double helical DNA.

In the PS spectra the natural intuitions mentioned above are experimentally demonstrated; neither the 1222 or 1065 cm^{-1} canonical bands are present. There is, however, a hydration sensitive mode at 1156 cm^{-1} at 0% RH which softens 11 cm^{-1} by 98% RH. This behavior is reminiscent of the 1245 to 1222 cm^{-1} shift with the same hydration change in double helical (16,17) and triple helical (19) DNA. There are three other noteworthy features in this region: 1006 and 1056 cm^{-1} bands and a hydration dependent 'slope'—a relatively straight horizontal line presumably the result of several absorptions of equal intensity and slightly different frequencies—between the nominal 1156 and 1056 cm^{-1} bands. The 1006 and 1056 cm^{-1} bands appear much stronger than putative corresponding bands in the 75% RH PO oligodeoxyribonucleotide spectrum. Grouping these two features produces one of the strongest candidates of a PS oligodeoxyribonucleotide signature in the mid-infrared. The 1210 cm^{-1} band in the PS spectra are possibly a mode which has attracted little attention until now because of its diminutive size relative to the asymmetric stretch at 1222 cm^{-1} in most double helical DNAs. Since that asymmetric mode is absorbing elsewhere in PS the 1210 cm^{-1} has a clear presence. Perhaps for similar reasons the 1250 cm^{-1} band does not have a corresponding band in the PO oligodeoxyribonucleotide spectra.

Glycosidic frequency region

This region hosts bands which are the result of atomic motions between the bases and their corresponding sugars. The 1286 cm^{-1} band in the PO spectra is hydration insensitive and relatively much more intense than the corresponding A-form marker band at 1275 cm^{-1} in adenine containing double helical polynucleotides (16). The next higher mode at 1325 cm^{-1} has little hydration dependence whereas in double helical DNA this band splits to 1335 and 1317 cm^{-1} (16). The 1375 cm^{-1} mode indicates the *anti* conformation of the glycosidic bond (18). The nominal 1425 cm^{-1} band is a signature for the A, B and Z conformations of DNA (18), and here the actual frequency of 1424 cm^{-1} indicates A conformation of the deoxyribose moiety and confirms the *anti* conformation. Between 1424 and 1482 cm^{-1} in the PO spectra there is another nearly horizontal line-like section of the spectrum similar to the frequency region of 1056–1156 cm^{-1} in the PS spectra—this spectral characteristic also occurs in double helical DNA at all RHs (16). In both cases it would be possible to use Fourier self-deconvolution to help resolve the bands which comprise these regions, but doing so is beyond the scope of the present treatment.

PS spectra in the glycosidic region exhibit some similarities and several significant differences with respect to PO. The 1291 cm^{-1} band corresponds to the 1286 cm^{-1} band in PO and is equally hydration insensitive, but appears to have a smaller half-width at half-height. The 1325 cm^{-1} band in PS is much weaker than in PO at all RHs. The band indicating glycosidic *anti* conformation, 1369 cm^{-1}, is present at all RHs in both PS and PO. It is noteworthy that this robust hydration insensitivity in single-stranded oligodeoxyribonucleotides is in sharp contrast with its extreme hydration sensitivity in higher order DNA structures such as triple helices (19). As mentioned above the nominal 1425 cm^{-1} band is a conformation marker depending on its exact frequency; in PS oligodeoxyribonucleotides it is at 1415 cm^{-1} which indicates that, at least the atoms involved in this absorption—if not the entire molecule—are in a Z-like conformation. The original assignment of Z conformation for the 1413 cm^{-1} band was made using X-ray and IR data on GC-rich sequences that had runs of cytosine bases as does the sequence used in this study (20). Finally, there is no absorption to speak of—in comparison to the PO spectrum—between the frequencies 1415 and 1490 cm^{-1}. The assignment of a 1460 cm^{-1} band in double helical DNA to coupled adenine sugar vibrations (21) seems to be called into question by a comparison of this frequency neighborhood in PO and PS samples presented in Figures 2 and 3, respectively. The question being, at least, how does the substitution of sulfur for a non-bridging oxygen abrogate the adenine-sugar absorption?

Base stacking frequency region

In this frequency region the half-width at half-height of the PO bands are large enough to reduce the absorption to shoulders instead of bands with maxima. The 1529 cm^{-1} band and a minuscule spectral feature at nearly 1545 cm^{-1} in the 33% RH spectra form a non-Lorentzian shaped band at higher RHs. The 1578 and 1608 cm^{-1} bands are only perceptible at low RHs; 0% RH being best. At slightly higher frequencies the set of absorptions nearly appear as one very broad absorption centered at 1654 cm^{-1}. The absence of a band at 1715 cm^{-1} (C2=O2) in both PO and PS spectra indicates a lack of base stacking at all RHs (17). This evidence confirms the physical intuition that single-strands would not have much base stacking due to the absence of hydrogen bonding and the hydrophobic nature of the nitrogenous bases. In other words, the van der Waals interactions of base stacking are insufficient to overcome the single-strand backbone conformational energy required for collective alignment of the bases. The PO spectra have a 1725 cm^{-1} shoulder which is a signature of the guanine C6=O6 Hoogsteen binding (18).

This frequency region supports the contention that the bands in the PS spectra are sharper than the corresponding bands in the PO spectra. In particular the 1606 cm^{-1} band is well resolved in PS, but much less so than the corresponding 1608 cm^{-1} band in PO. Similar comments apply to the 1653 cm^{-1} band of PS. In pronounced contrast with the PO spectra in this frequency region, the PS spectra have no evidence of a band at 1725 cm^{-1}. This spectral difference maybe tentatively explained by the PS oligodeoxyribonucleotide's inability to form higher order structures, such as triple helices, which require Hoogsteen binding.

Through the comparison of mid-infrared observations of the hydration-dependent structure of a PS oligodeoxyribonucleotide and an unmodified phosphodiester parent it is clear that, questions of stereo-chemical purity aside, there are significant structural differences at all relative humidities and important hydration dependent spectroscopic behaviors within each sample. The best example of intra-sample hydration behavior is the ν_{as} PO_2 band at 1222 cm^{-1} does NOT undergo a frequency shift to 1245 cm^{-1} as hydration decreases, as is the case with duplex DNA. The principle spectroscopic difference between samples is likely to be the 656 cm^{-1} band which is prominent in PS and absent in PO spectra. The PS bond is not well characterized, and there is considerable debate over charge distribution, bond order and bond length; this is currently an area of active research (22–25). Spectroscopic experiments on model compounds and their isotopic derivatives are presently in progress in order to clarify the role of the PS bond in the oligodeoxyribonucleotide backbone toward the goal of providing fundamental data for molecular dynamics and MSPA calculations (11) are in progress.

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