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Biophysical Chemistry 119 (2006) 1 – 6

Biophysical Chemistry

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Vibrational circular dichroism signature of hemiprotonated intercalated four-stranded *i*-DNA

D. Tsankov [a,c], M. Krasteva [b,c], V. Andrushchenko [c], J.H. van de Sande [c,d], H. Wieser [c,*]

[a] *Institute of Organic Chemistry, Bulgarian Academy of Sciences, BG-1113 Sofia, Bulgaria*
[b] *Department of Biochemistry and Biophysics, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden*
[c] *Department of Chemistry, Faculty of Science, 2500 University Drive NW, University of Calgary, Calgary, AB, Canada, T2N 1N4*
[d] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, Heritage Medical Research Bldg., 3330 Hospital Drive N.W., University of Calgary, Calgary, AB, Canada, T2N 1N4*

Received 16 May 2005; received in revised form 17 August 2005; accepted 17 August 2005
Available online 23 September 2005

Abstract

The four-stranded intercalated DNA structure exemplified by the oligonucleotide 5′-d(CCCCCCCCCCCC) ($d(C)_{12}$) was studied at acidic pH by infrared absorption (IR) and vibrational circular dichroism (VCD) spectroscopy and compared with spectra of the same oligonucleotide at neutral pH to establish distinct VCD markers for the intercalation motif. The most striking feature is a new absorption at 1694 cm^{-1} and its corresponding VCD couplet with reversed sign. These are unique for the intercalated structure and have not been observed for other parallel stranded duplexes. Significant characteristic features resulting from the spatial arrangement of the sugar-phosphate backbone are also clearly present for $d(C)_{12}$ at acidic pH. An extensive network of CH. . .O bonds twists the backbone such that multiple through-space vibrational coupling occurs among neighbouring sugar-phosphate residues resulting in unusual VCD signals.

Keywords: *i*-DNA; 5′-d(CCCCCCCCCCCC); Four-stranded DNA structure; Vibrational circular dichroism spectroscopy; Infrared absorption spectroscopy

1. Introduction

Over 40 years ago it was discovered that DNA containing stretches of cytidine residues could form parallel stranded duplexes at acidic pH stabilized by hemiprotonated CH^+·C base pairs [1]. Solution and crystal studies carried out mostly on polycytidilic acid at pH 4.5–6.5 confirmed the proposed base pairing, but all results were interpreted by assuming a regular double stranded structure [2–5]. A later NMR study on $d(TC_5)$ suggested an unusual structure in which two parallel-stranded duplexes held by CH^+· C base pairs intercalate with each other in an antiparallel (opposite strand) orientation to form a four-stranded complex (Figs. 1 and 2) [6]. The DNA tetramers were designated as *i*-motifs with reference to intercalating parallel stranded duplexes. The C-quadruplexes are referred to most often as *i*-DNA. NMR data also revealed that in solution the DNA tetrads posses a slow right-handed helical twist with about 16° between base pairs belonging to the same duplex. Further details of this DNA structure have been proposed after systematic NMR and X-ray diffraction studies of short oligonucleotides containing stretches of cytosine [7–11]. Another significant structural aspect of the intercalated duplexes is the rise of about 6.2 Å between neighbouring residues, and planar base pairing between cytosines protonated at the N3 position. As a consequence of its geometry, *i*-DNA differs substantially from the known B-, A- or Z-forms of double-stranded DNA as well as from the guanine tetrads [12,13]. The unusual form of the *i*-motif affects also the base stacking. Unlike the normal 3.4 Å distance found in nucleic acids, the inter-base distance averages 3.1 Å between adjacent cytosine residues along the axis [8]. Moreover, the neighbouring pyrimidine rings do not overlap. Instead, the exocyclic carbonyl and amino groups stack on top of each other [6,8]. Another unique structural characteristic of *i*-DNA is that the intermolecular contacts among sugar-phosphate backbones are abnormally close reaching 5.9 Å interstrand *P*–*P* distances

* Corresponding author. Tel.: +1 403 220 4934; fax: +1 403 289 7635.
E-mail address: hwieser@ucalgary.ca (H. Wieser).

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doi:10.1016/j.bpc.2005.08.004

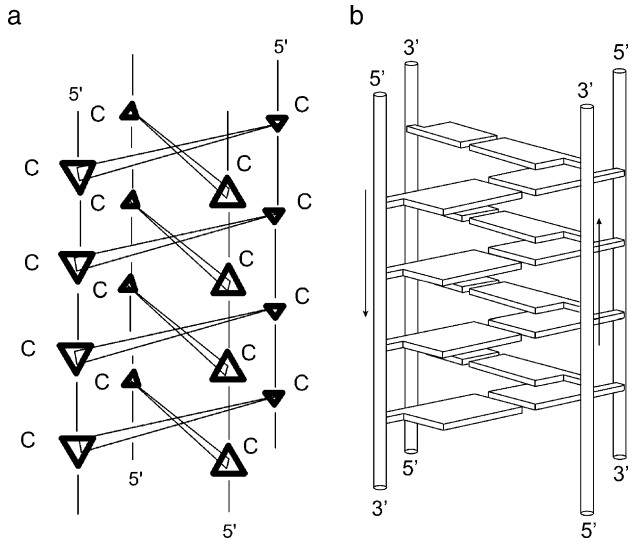

Fig. 1. (a) Fragment of intercalation motif assumed for 5′-d(CCCCCCCCCCCCC) (d(C)$_{12}$). (b) Schematic representation of the intercalation motif for 5′-d(CCCCCCCCCCCC) (d(C)$_{12}$) (after Gehring et al. [6]).

[10]. It was recently found that the close proximity between pairs of antiparallel oriented backbones is a consequence of stabilizing C–H. . .O hydrogen bonding between neighbouring deoxyribose sugars [14].

The p*K* value governing cytosine N3 protonation is 4.2, i.e., more than two units lower than the physiological pH. The biological relevance of this structure has not been considered until it was found that C-quadruplexes could exist at slightly acidic and on some occasions even at neutral pH [15]. Furthermore, the complexes are stable at higher pH values. These observations suggested a relatively high probability that such a structure could exist under physiological conditions. The novel C-quadruplex is also of biological interest because cytosine-rich sequences occur naturally as a complement to guanine-rich sequences in telomeric DNA as well as in some non-coding regions of eukaryotic DNA such as promoter sites, satellite DNAs and introns [16].

Vibrational circular dichroism (VCD) spectroscopy has proved to be a reliable method for studying DNA conformations and structural phase transitions in solution. Being an infrared analogue of electronic circular dichroism, VCD combines the advantages of both methods. VCD is as stereoselective in the infrared due to characteristic vibrational modes as electronic CD is in the ultraviolet due to electronic transitions. In nucleic acids the VCD signals arise from through-space vibrational coupling of identical oscillators arranged in a helical formation reflecting base sequence and specific order. The corresponding features have positive and negative components referred to as “couplets”. The high sensitivity of these signals to any change in vibrational coupling resulting from changes in base sequence, molecular geometry or molecular conformation leads to appreciable changes in the spectra. Thus, VCD is capable of providing unique structural information that is not accessible by infrared, Raman or electronic CD, but in principle can complement other solution techniques such as NMR. VCD has been particularly useful in recent studies of structural transitions of nucleic acids between B- and Z-form, duplex-triplex-single strand transformation, DNA condensation and aggregation, and DNA interactions with metal ions and with the anticancer drug cisplatin [17].

As a part of a larger project aiming to ascertain VCD markers as diagnostics for DNA structure in diverse structural forms [18], we investigated the *i*-motif with d(C)$_{12}$ as the target sequence at different pH values. Others have previously examined four-stranded *i*-DNA of d(CCCT) and d(C)$_8$ in the crystal form and in solution by Raman spectroscopy [19]. Although some Raman markers have been identified, the carbonyl region of the spectra appeared not as informative as some other regions.

2. Materials and methods

The d(C)$_{12}$ oligonucleotide was synthesized in the University of Calgary Core DNA Services (Applied Biosystems Model 380B) by the standard phosphoamidite method. The sample was extensively desalted and purified by Sephadex G-25 packed column chromatography, eluted with double distilled water and lyophilized to dryness. A test to assess the purity of the oligonucleotides was conducted by polyacrylamide gel electrophoresis. The purified oligomer was dissolved in D_2O, and the solution was stabilized by adding 100 mM NaCl. The sample was further lyophilized and re-dissolved three times in 99.5% D_2O (Aldrich) in order to achieve complete deuterium exchange. The pH was fixed at 7.2 by adding 0.1 N NaOD for the initial measurement. At lower pH the VCD measurements were carried out after titrating the solute with 0.1 N DCl.

A Bomem MB100 spectrometer equipped with VCD optics described elsewhere was used for measuring the spectra [20]. The concentration of d(C)$_{12}$ in D_2O was 10 mM/strand. All samples were contained in a standard 50 μm BaF_2 cell (International Crystal Laboratories) maintained at 5 °C in a variable temperature chamber with a circulating water thermostat (NESLAB Instruments Inc.) and monitored by a Cu-constantan thermocouple (OMEGA Technology Company Inc.) with an accuracy of ±0.1 °C. The VCD spectra were generated by accumulating 7500 ac scans (105 min collection time) and ratioed against 750 dc scans at 4

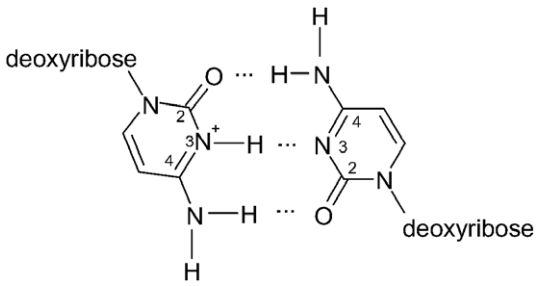

Fig. 2. Scheme of hydrogen bonding between hemiprotonated CH$^+$·C base pairs.

cm^{-1} resolution to achieve good signal-to-noise ratio and flat baselines. The spectra were further corrected for absorption and polarization artifacts by subtracting the solvent VCD spectra taken at the same conditions prior to sample measurements.

3. Results and discussion

The infrared absorption (IR) and VCD spectra at 5 °C of the target sequence $d(C)_{12}$ at pH 7.2, 5.6 and 3.8 are displayed in Figs. 3 and 4, respectively, and of poly(rC) at pH 6.2 in Fig. 5. The corresponding wavenumbers are listed in Table 1.

3.1. In-plane base vibrations of $d(C)_{12}$ at different pH

Of particular interest is the region of 1700–1450 cm^{-1} since it contains the prominent vibrational bands arising from carbonyl stretching and specific in-plane ring modes of the nucleic bases. The changes in base pairing and base stacking induced by N3 protonation are expected to cause concomitant differences in the IR and the VCD spectra.

Similar to poly(rC) and poly(dC), which at neutral pH form single stranded structures with stacked bases and ordered backbones [21,22], $d(C)_{12}$ also shows elements of well ordered single strands (designated here as s-$d(C)_{12}$). The single strong absorption of $d(C)_{12}$ at 1650 cm^{-1} arises from stretching of the C2═O carbonyl group (pH 7.2 in Fig. 3) [23,24]. Other absorptions of interest are at 1614 cm^{-1}, assigned to a ring stretching mode of cytosine [18,25], and those appearing prominently at 1523 and 1506 cm^{-1} originating from different

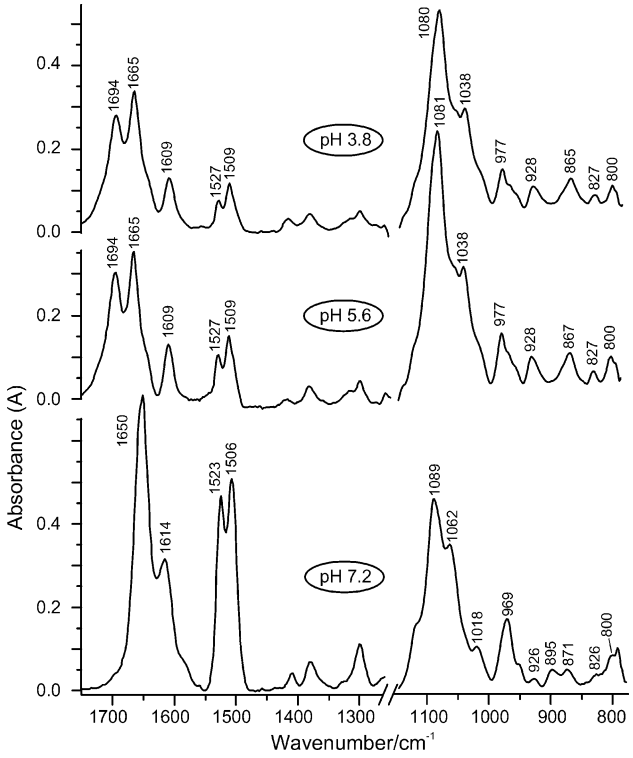

Fig. 3. Infrared absorption spectra of $d(C)_{12}$ at pH 7.2, 5.6 and 3.8 in D_2O, 10 mM $d(C)_{12}$, 50 μm BaF_2 cell, 5 °C and 4 cm^{-1} resolution.

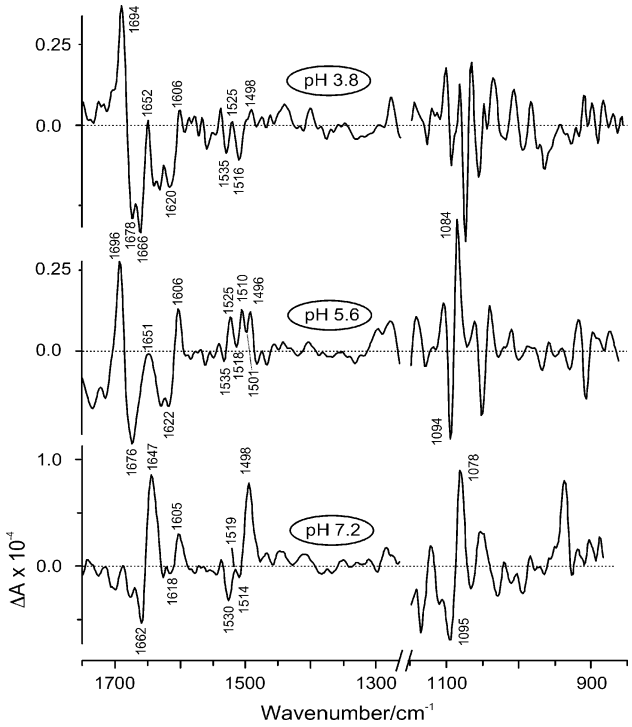

Fig. 4. VCD spectra of $d(C)_{12}$ at pH 7.2, 5.6 and 3.8 in D_2O, 10 mM $d(C)_{12}$, 50 μm BaF_2 cell, 5 °C and 4 cm^{-1} resolution.

pyrimidine skeletal modes of which the former has been ascribed to a ring mode of free cytosine involving mostly C═N3 stretching, which is sensitive to Watson–Crick base pairing [24,26,27].

At neutral pH s-$d(C)_{12}$ has an intense couplet at 1662(−)/1647(+) cm^{-1} (1664(−)/1646(+) cm^{-1} in poly(rC) at pH 6.2, Figs. 4 and 5, respectively), which without doubt is associated with C2═O carbonyl stretching. Another significant feature in

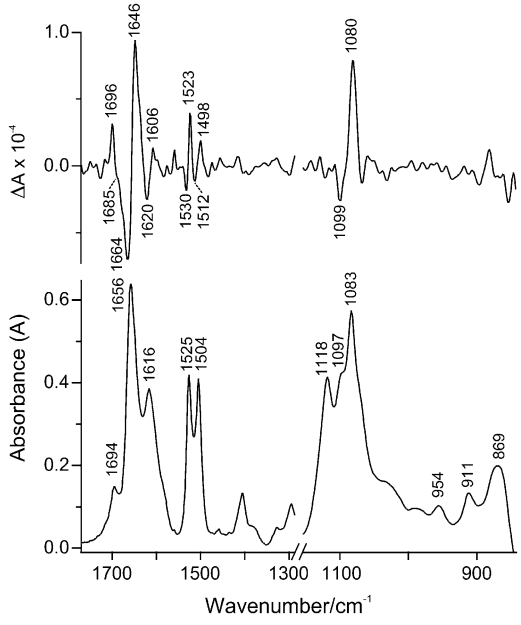

Fig. 5. Infrared absorption (bottom) and VCD spectrum (top) of poly(rC) at pH 6.2 in D_2O, 5 °C and 4 cm^{-1} resolution.

Table 1
Absorption and VCD peak positions (cm^{-1}) of $d(C)_{12}$ at neutral pH (s-$d(C)_{12}$) and at low pH (*i*-$d(C)_{12}$)

	s-$d(C)_{12}$ pH 7.2		*i*-$d(C)_{12}$ pH 5.6		*i*-$d(C)_{12}$ pH 3.8		poly(rC) pH 6.2	
C2═O CH^+·CH			1694	1696(+)/1676(−)	1694	1694(+)/1678(−)	1694	1696(+)/1685(−)
			1665	1651(+)	1665	1666(−)/1652(+)		
C2═O cytosine	1650	1662(−)/1647(+)					1656	1664(−)/1646(+)
C═C ring mode	1614	1618(−)/1605(+)	1609	1622(−)/1606(+)	1609	1620(−)/1606(+)	1616	1620(−)/1606(+)
C═N cytosine	1523	1530(−)/1519(+)	1527	1535(−)/1525(+)	1527	1535(−)/1525(+)	1525	1530(−)/1523(+)
C═C, C═N cytosine	1506	1514(−)/1498(+)	1509	1518(−)/1510(+)	1509	1516(−)	1504	1512(−)/1498(+)
				1501(−)/1496(+)		1498(+)		
PO_2^- sym stretch	1089	1095(−)/1078(+)	1081	1094(−)/1084(+)	1080		1083	1099(−)/1080(+)
Sugar modes	1062		1038		1038			
	1018							
	969		977		977			
	926		928		928			
	895							
	871		867		865			
	826		827		827			
	800		800		800			

this region is the positive peak at 1605 cm^{-1}, which while corresponding to the 1614 cm^{-1} absorption, shows but a week negative component at 1618 cm^{-1}. The corresponding couplet is clearly visible at 1620(−)/1606(+) cm^{-1} in poly(rC). At neutral pH, the counterparts of the absorptions at 1523 and 1506 cm^{-1} appear as positive couplets at 1530(−)/1519(+) and 1514(−)/1498(+) cm^{-1}. They are in almost the same position in poly(rC), although the broader VCD features in $d(C)_{12}$ suggest a slightly altered helical structure.

Upon decreasing the pH, substantial changes occur in both absorption and VCD due to CH^+·C base pair formation. According to the proposed four-stranded intercalated structure [6], the base paired strands are parallel to each other and both parallel duplexes are oriented in an antiparallel fashion (Fig. 1). The whole structure is held by hemiprotonated base pairs where the hydrogen bonds are formed between the C2═O groups and the C4–ND_2 groups of the paired cytosine moieties. The third hydrogen bond occurs between the N3 atoms (Fig. 2). The quarternization of N3 next to the carbonyl groups leads to a re-distribution of the electron density in the whole molecule shifting more π-electron density into the carbonyl groups thereby affecting the force constants of all bonds in which π-electrons are involved, particularly carbonyl stretching [6]. Consequently, the IR and VCD spectra of the protonated species at pH 5.6 and 3.8 are similar to one another (Figs. 3 and 4). The carbonyl absorption of s-$d(C)_{12}$ at neutral pH splits into two bands, which are shifted to higher wavenumbers for the paired cytosines by protonation of N3 from 1650 cm^{-1} at pH 7.2 to 1694 and 1665 cm^{-1} at lower pH [22,26]. The ring mode is shifted from 1614 to 1609 cm^{-1} with somewhat lower intensity. The absorptions at 1523 and 1506 cm^{-1} move only slightly to 1527 and 1509 cm^{-1} and are accompanied by a considerable loss of intensity. The VCD spectra are changed much more significantly. At pH 5.6, the main VCD couplet occurs as a relatively broad feature at 1696(+)/1676(−) cm^{-1} with opposite sign and corresponds to the 1694 cm^{-1} absorption (cf. Figs. 3 and 4). At pH 3.8, the related VCD feature is clearly resolved into two components at 1694(+)/1678(−) cm^{-1} and 1666(−)/1652(+) cm^{-1}. The 1618(−)/1605(+) cm^{-1} VCD couplet at neutral pH appears to be preserved at 1622(−)/1606(+) and 1620(−)/1606(+) cm^{-1} at pH 5.6 and 3.8, respectively, with the corresponding absorption shifted from 1614 to 1609 cm^{-1}. In both acidic conditions, the pyrimidine skeletal modes at 1527 and 1509 cm^{-1} display confusing and description-defying VCD features. A couplet at 1535(−)/1525(+) occurs at both pHs, while the 1518(−)/1510(+) cm^{-1} feature is clearly evident at pH 5.6 but survives as a negative peak at 1516 cm^{-1} at pH 3.8. The additional couplet at ~1501(−)/1496(+) cm^{-1} with pH 5.6 and the positive remnant at 1498 cm^{-1} at pH 3.8 can be ascribed to the similar vibration in the unprotonated oligomer at 1514(−)/1498(+) cm^{-1}.

The IR and VCD spectra of poly(rC) confirm the foregoing assignments (Fig. 5). At pH 6.2, a small fraction of cytosine residues is protonated as the shoulder in IR at 1694 cm^{-1} and the relatively weak couplet at 1696(+)/1685(−) cm^{-1} in VCD hidden under the intense couplet at 1664(−)/1646(+) cm^{-1} suggest. The negative lobe of this latter VCD feature originates from C2═O stretching of the unprotonated cytosine residues with corresponding principal absorption at 1656 cm^{-1}. All the remaining absorption and VCD bands appear at the same wavenumbers as those of the unprotonated $d(C)_{12}$ and agree well with those of unprotonated poly(rC) at neutral pH [22].

In the four-stranded intercalated complex the neighbouring base pairs are arranged in such a way that no overlap exists among the charged six-membered aromatic rings [6,8]. Instead, the stabilization effect is achieved at the expense of dipole–dipole interactions between pairs of exocyclic carbonyl and amino groups, which are oriented in an antiparallel fashion [14]. This specific orientation of the neighbouring base pairs most likely serves to avoid destabilizing interactions between the positively charged ring systems and is the main reason for the sign reversal observed in the VCD spectra.

3.2. PO_2^- and sugar modes

The absorption spectra in the range 1100–770 cm^{-1} of the free single strand, s-$d(C)_{12}$, and the self-associated intercalated

complexes of *i*-d$(C)_{12}$ recorded in D_2O at different pH are compared in Fig. 3. The major band at 1089 cm^{-1} at neutral pH originates from the symmetric PO_2^- stretching vibration [23,27]. Other bands in this region, namely those at 1062, 1018, 969, 926, 895, 871, 826 and 800 cm^{-1}, belong to different sugar modes. The 865 and 800 cm^{-1} absorptions at acidic pH are IR marker bands for N-type sugars, while an absorption at 835 cm^{-1} has been identified as arising from S-type sugars [27].

It has been deduced by NMR for 5′-d(TCCCCC) that the sugar-phosphate backbones of the four-stranded complex are pair-wise associated bringing the sugar rings in sufficiently close proximity to create van der Waals bonds [6]. The pseudo rotational angle evaluated from these data indicated that the first and second deoxycytidine residues in 5′-d(TCCCCC) have C3′-endo sugar puckers while the remaining C-residues largely adopt C4′-exo conformation [6]. Further evidence for intermolecular hydrogen bonding between CH1′...O4′ of neighbouring sugars was reported subsequently by X-ray [12]. Performed on the shorter oligonucleotide d(TCCC), this study proposed a different distribution of the sugar conformations. Among the 12 residues of the quadruplex, six were identified as C3′-endo and two C2′-endo, while four adopted an envelope structure in which both C2′ and C3′ atoms are on the same side of the ring [12]. IR evidence supporting simultaneous co-existence of N and S type sugar puckering was also reported in a subsequent solution assay [26]. More recently, the stabilizing effect of the CH...O hydrogen bonds was examined experimentally and theoretically [28,29]. The NMR results and the molecular dynamics simulations carried out on model C-tetrads determined that the optimal backbone twisting essential for base pairs stacking and the stability of the whole complex is facilitated by closer sugar–sugar contacts. The molecular dynamics simulations favoured N-type sugar puckering, which assists in tighter sugar interactions [29]. Our absorption data also indicate that both C3′-endo and C2′-endo sugar puckering occur in solution. This assumption is based on the simultaneous appearance of the absorptions at 867 and 800 cm^{-1} characteristic for N-type and the band at 827 cm^{-1} typical for S-type sugars.

One further unique feature for the tetrameric structure is the absence of the absorption at 895 cm^{-1}. It is assigned as a deoxyribose vibration, which is persistently observed in the spectra of the unprotonated species [22,26]. Moreover, it is present in all spectra of DNA duplexes no matter whether they are parallel or antiparallel [19,22,24,26,27]. Some intensity decrease of this band has been reported only for the spectra of C^+·G·C triplets at acidic pH [22].

One significant spectral characteristic of the four strand intercalated complex is the shift of the major absorption of symmetric PO_2^- stretching from 1089 to 1081 cm^{-1}. This band is invariably situated around 1090 cm^{-1} in absorption of all DNA double helices at normal or acidic pH in parallel as well as antiparallel strands [19,22,24,26,27]. This peculiarity was first noticed in IR of d(TCCCCC) at acidic pH and was explained by a rotation of the phosphate groups stabilized by bridging water molecules [26].

The VCD spectra of d$(C)_{12}$ at the acidic pHs display a complicated profusion of strong couplet-like features between 1100 and 900 cm^{-1} (Fig. 4), which are difficult to assign in detail. Normally, a distinctive and dominant couplet arises in this region from the symmetric stretching vibration of PO_2^- in DNA as well as nucleic acid oligomers generally examplified here by the unprotonated d$(C)_{12}$ at pH 7.2 (Fig. 4). Instead, a distinct couplet remains at 1094(−)/1084(+) cm^{-1} for pH 5.6 together with a few other similar real features, whereas no definitive assignments are feasible intuitively for the VCD spectrum with pH 3.8. Apparently, through-space vibrational coupling among entities of the sugar-phosphate backbone still occurs. The systematic network of C–H...O hydrogen bonds involving C1′, C4′ and O4′ atoms, as reported in an X-ray study [14], appears to force the backbone into a conformation atypical for known DNA forms.

4. Conclusions

At acidic pH the four-stranded intercalated structure of the dodecamer 5′-d(CCCCCCCCCCCC) shows distinctive changes in infrared absorption and vibrational circular dichroism. The protonation at N3 and the formation of the C^+·C base pairs lead to substantial π-electron density re-distribution, which affects most of the bands in the spectra. Most noticeable in the carbonyl region are the two carbonyl absorptions shifted to higher wavenumbers. Other bands, which involve mainly in-plane ring vibrations of the bases with marked participation of C═N3 stretching, also shift to higher wavenumbers accompanied by a noticeable loss of intensity, whereas the ring mode at 1614 cm^{-1} shifts to lower wavenumbers. More explicit changes occur in the corresponding VCD spectra. One of the surprising features in VCD is the reversed sign of the main C2═O stretching couplet of the protonated species. It implies specific reorientation of neighbouring base pairs, which affects the stacking interactions of the carbonyl groups. In order to avoid the repulsion between the charged pyrimidine rings and therefore their destabilizing effect on the whole molecule, the *i*-DNA motif adopts a structure in which the six-membered aromatic rings do not overlap directly. Instead, the exocyclic carbonyl and amino groups stack in an antiparallel orientation [6,8] thereby affecting the sign of the VCD couplet.

The spectra in the 1100–800 cm^{-1} region, where the sugar-backbone vibrations occur, appear to change substantially when the C-tetrads are formed. The major absorption peak at 1089 cm^{-1} due to PO_2^- is shifted to 1081 cm^{-1}. Such wavenumber shifts have not been observed in either parallel or antiparallel duplexes regardless of the sequence. Evidently, these features are due to the intercalated structure. The absorption data also confirm that both C3′-endo and C2′-endo sugar puckers are present, which agrees with other solution data obtained by NMR, Raman and IR [6,19,26,28]. Another characteristic feature of the four-stranded intercalation motif is the specific backbone conformation. The close distance between base paired backbones, where the interstrand $P–P$ distances are even shorter than the intrastrand distances between adjacent phosphorus atoms, favours the formation of

interstrand CH. . .O bonds [6]. Indeed, an extensive network of CH. . .O bonds involving C1′, C4′ and O4′ was established [14], which twists the backbone such that multiple through-space vibrational coupling occurs among neighbouring sugar-phosphate residues resulting in unusual VCD signals in this spectral region.

At the current stage of development and owing to its enhanced stereosensitivity, VCD can provide other perspectives for structural details. Notwithstanding its limitations, VCD is clearly capable of supplying structural data, which are complementary to other solution techniques.

Acknowledgements

The provision of funding from the Natural Sciences and Engineering Research Council to H. Wieser for the conduct of this research is most gratefully acknowledged.

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