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On the influence of conformational locking of sugar moieties on the absorption and circular dichroism of nucleosides from synchrotron radiation experiments

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Abstract

Absorption and synchrotron radiation circular dichroism (SRCD) spectra of nucleosides and locked nucleosides containing either the adenine and thymine nucleobase were measured in the ultraviolet and vacuum ultraviolet (down to 176 nm) regions. The CD spectra strongly depend on the chemical structure of the sugar and the introduction of a methylene bridge that locks it into a specific conformation. Spectra of compounds with different configurations at the sugar atoms shed light on what determines the sign and intensity of the CD bands.

Keywords: Synchrotron radiation; Circular dichroism; Absorption spectroscopy; Vacuum ultraviolet; Locked nucleosides

1. Introduction

Circular dichroism (CD) spectroscopy is extensively used in conformational analysis of optically active biological molecules, such as proteins, peptides and nucleic acids in the solution phase [1–6]. Nucleic acid duplexes of A and B type structures exhibit characteristic positive and negative peaks at specific wavelengths in the ultraviolet region of their CD spectra. A change in relative intensity of these peaks due to chemical modification at any nucleotide site is qualitatively related to changes in A–B type structural pattern [7]. In general, the overall structure of the nucleic acid duplexes is determined by conformations of the flexible ribose/deoxyribose sugar rings [8]. To understand the origin of the CD spectral changes due to chemical modifications at specific sites of nucleic acid duplexes, it is essential to investigate the spectra of modified nucleotides.

Nucleic acids in which one or more of the nucleotide sites are altered incorporating methylene bridges between O2′ and C4′ atoms of their ribose sugars, popularly known as locked nucleic acids (LNAs) [9], have attracted a lot of attention in recent years [10–13]. Oligonucleotides containing such modified sugar moieties exhibit remarkable helical thermal stability when hybridized to complementary DNA or RNA without detectable reduction in sequence selectivity [14–16]. Furthermore, the melting temperature of a modified duplex is enhanced by about 4–9 °C for incorporation of each modified nucleotide compared to unmodified duplexes [16]. Although CD spectroscopy in the UV region (shorter wavelength limit ∼190 nm) along with proton NMR spectral data has been used to investigate the conformations of LNA:DNA duplexes [17,18], to our knowledge the effects of such methylene locking in the sugar moiety on the CD spectral features of individual nucleosides have not been investigated so far.

In a recent paper we demonstrated that CD spectroscopy in the vacuum ultraviolet (VUV) region using synchrotron radiation (SR) provide unprecedented detailed signatures of the conformational changes of sugars, nucleosides and mononucleotides

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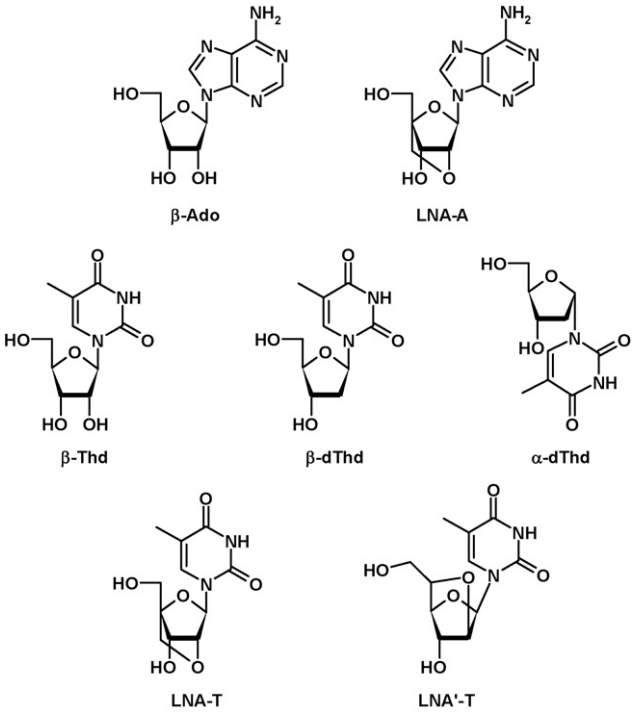
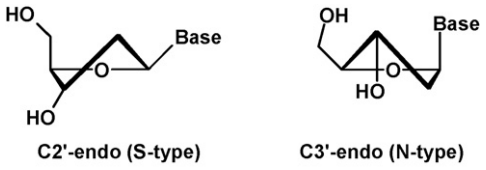


Scheme 1. Structures of the compounds under study.

due to changes of pH and temperature of the medium [19]. The advantage of synchrotron radiation for such experiments is the large available photon fluxes in the VUV where absorption is strong [20]. The current work is a continuation of this theme with the goal of obtaining a fundamental understanding of the governing factors for VUV CD bands of the basic building blocks of DNA and RNA. Nucleosides have been extensively studied by circular dichroism together with calculations of rotational strengths demonstrating large dependences on sugar conformation and rotation about the glycosidic bond on the UV CD bands [21–29]. However, there is sparse literature on the spectral signatures in the VUV region [19,28]. Here we report absorption and SRCD spectra of nucleosides having purine (adenine) and pyrimidine (thymine) bases, and we analyze the spectral effect for incorporation of methylene internal locking in the sugar moiety between O2′ and C4′ and between O2′ and C5′. The compounds chosen for study are shown in Scheme 1. The importance of the nucleobase being in its α or β position (nucleobase down or up relative to the C5′ atom of the sugar ring orientation in Scheme 1) was also investigated for thymidine (dThd). In the case of unlocked sugars, there is an equilibrium between the C2′-endo and C3′-endo furanose conformations, cf. Scheme 2,



Scheme 2. Furanose conformations.

whereas the locked sugars are fixed in C3′-endo (LNA-A and LNA-T) and C2′-endo (LNA-T′) type conformations.

2. Experimental part

Absorption and SRCD spectra were collected on beamline UV1 at ASTRID, part of the Institute for Storage Ring facilities, the University of Aarhus, Denmark [30,31]. The beamline was calibrated for wavelength and optical rotational magnitude at the beginning of each fill of the storage ring (once a day). Adenosine (ade) and thymidine (dThd) were purchased from Sigma–Aldrich. Locked forms of the nucleosides were synthesized according to Ref. [9b,c]. All compounds were on solid form. Known amounts of compounds (measured on a scale) were dissolved in deionized water or 10 mM phosphate buffer to a concentration of 5 mM. Measurements were done at pH 2, 6–7, and 10. Spectra for baseline subtraction were recorded of deionized water, 5 mM NaH_2PO_4/5 mM Na_2HPO_4, and 5 mM Na_2HPO_4/5 mM Na_3PO_4. Samples were measured in 100-μm path-length Suprasil open cells (Hellma GmbH & Co KG). In all cases at least five sample and three baseline spectra were collected at 20 °C using a dwell time of 2 s and with 1-nm step size. The limit for the lowest wavelength was determined by the absorption of water, the buffer, and the solute.

Also, for some of the compounds spectra were collected at several temperatures ranging between 5 and 90 °C. A spectrum at the initial temperature was recorded after the high temperature experiment to test for reproducibility. A 100-μm closed cell was used for the temperature dependent measurement to limit evaporation of the sample at high temperature.

All the spectra were averaged, baseline subtracted and slightly smoothed with a Savitzky-Golay filter using the CD data processing software CDTool [32].

3. Results and discussion

3.1. Absorption spectra

Absorption spectra of adenine nucleosides are shown in Fig. 1. The bands are predominantly due to strong $\pi\pi^*$ transi-

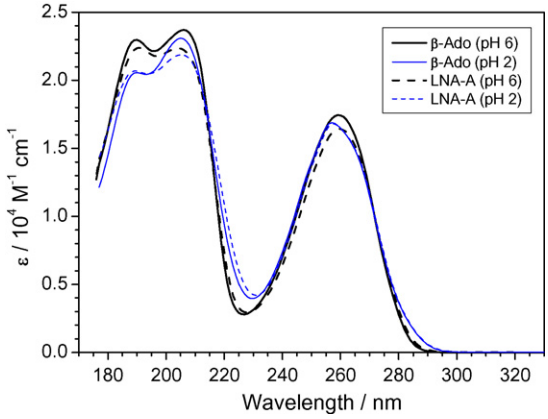


Fig. 1. Absorption spectra of adenine nucleosides at two different pH values (2 and 6). At pH 6, the adenine nucleobase is neutral whereas it is protonated at pH 2.

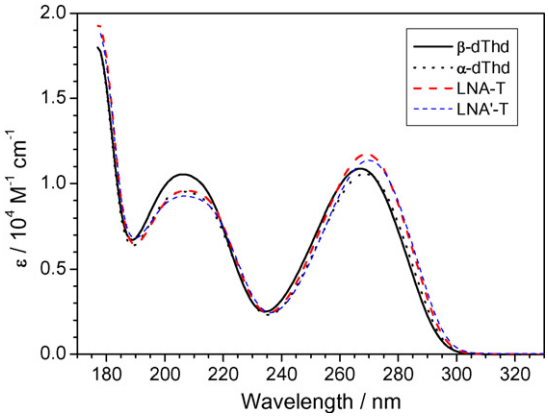


Fig. 2. Absorption spectra of thymine nucleosides in pure water (pH 6). The nucleobase is neutral.

tions in the nucleobase moiety [28,33], the $n\pi^*$ transitions being much weaker. It is evident from Fig. 1 that protonation of adenine only slightly changes the band maxima and the oscillator strengths: the 260-nm band is blueshifted to 257 nm whereas the bands centered at 205/206 and 190 nm maximally change by 1 nm. There is, however, a decrease of the latter two bands in intensity upon adenine protonation. In addition, spectra of locked forms are nearly identical to those of unlocked sugars. We note that the absorption of the phosphoric acid solution is insignificant compared to the absorption of the solutes: at the lowest wavelength limit of 177 nm where the absorption of the water and buffer are strongest, the absorption of water is almost two orders magnitude less than that of the solute and the absorption of the buffer is more than one magnitude less than that of the solute.

A comparison of the absorption spectrum of adenosine with that previously published for adenine [34] reveals that sugar substitution at the N9 position of the adenine ring via the glycosidic linkage causes significant perturbations on the electronic transition energies of the adenine molecule. Particularly, the band at 190 nm of adenosine results due to the presence of the sugar moiety since it is absent in the spectrum of the adenine base itself. The perturbation is more on the transitions belonging to the five-member imidazole ring, which appear at higher energies compared to the transitions belonging to the six-member ring, although the two rings are fused in the molecule [35].

In the case of locked and unlocked thymine nucleosides, the spectra are also quite similar with band maxima varying within 3 nm (Fig. 2). It plays hardly any role whether the anomer is β or α as evidenced from the spectra of α-dThd and β-dThd. In summary, nucleobase protonation, locking of the sugar, or changing the configuration between α and β have little effect on the absorption.

3.2. SRCD spectra of adenosine (β-Ado) and locked-sugar adenosine (LNA-A)

The SRCD spectrum of LNA-A in aqueous solution at pH 7 in the spectral range of 170–300 nm is presented in Fig. 3.

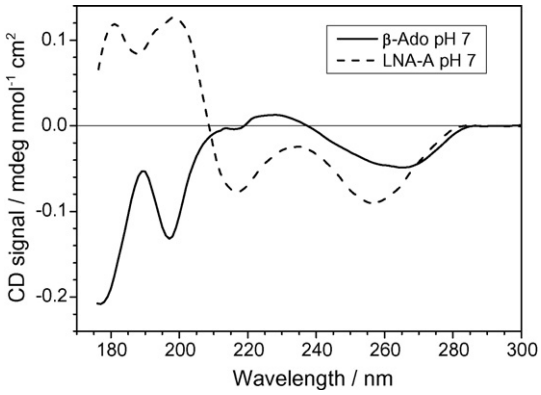


Fig. 3. SRCD spectra of β-Ado and LNA-A at pH 7.

The spectrum features with two intense positive bands in the VUV centered at 181 and 199 nm, and two negative bands having maxima at 217 and 257 nm. The negative band at 257 nm is very broad compared to the other bands. The spectrum of adenosine measured under the same experimental conditions (Fig. 3) is dramatically different from that of LNA-A. The longest wavelength band is affected least: the sign of the band remains the same, but the peak is about 10 nm redshifted. Earlier, this band has been proposed to be the overlap of at least three transitions [36,37], and because of locking the shorter wavelength components of the band appear to be more intense. The bands in the VUV region are affected to the largest extent, and the sign of the CD signals are reversed. The peak of the <178-nm negative band of adenosine appears about 5 nm redshifted in the locked nucleoside, and the weak negative band at 217 nm of the former turns into a large negative band in the spectrum of the latter.

As mentioned above, the methylene bridge in the sugar moiety does not have significant impact on the $\pi\pi^*$ transition energies of the base moiety, but the conformational change induced by such a bridge strongly affects the CD active bands.

In Fig. 4 we have presented a comparison of the CD spectra of LNA-A at four different pH values of the solutions. On lowering pH from 7 to 2, the longest wavelength broad peak remains almost unaltered. The negative band at 217 nm becomes slightly more intense and undergoes a redshift of about 2 nm. However, significantly large changes are noticed for the bands below 200 nm. The positive band at 198 nm is switched over to

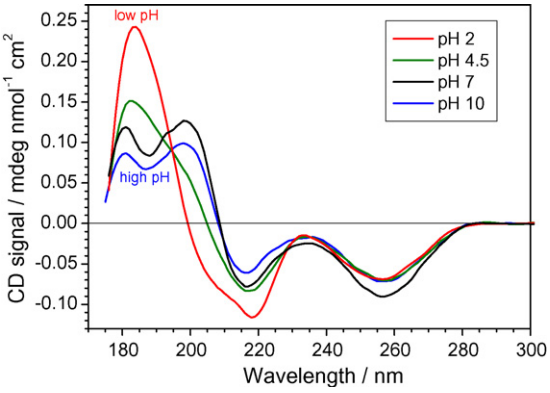


Fig. 4. SRCD spectra of LNA-A at different pH values.

an intense negative band, and the intensity of the 181-nm band exhibits about four-fold enhancement and a redshift of nearly 3 nm. This amazingly large pH sensitivity of these two CD bands indicates that their rotational strengths depend on the protonation state of the adenine ring. The spectrum at low pH of LNA-A also shows little resemblance with that previously published of β-Ado [19].

At pH 2, the ionized form of adenine dominates because the pK_a value of protonated adenine is ∼3.8. Since the energy of the nonbonding orbital is lowered on protonation, adenine protonation is likely to have the largest influence on the energies of the $n\pi^*$ transitions that involve a nitrogen lone pair. The CNDO-CI calculation of Hug and Tinoco [38], which considers only the valence electrons, predicts that the three highest energy nonbonding molecular orbitals of the adenine base consist of the lone-pair electrons on N1 and N3, N1 and N7, and N3 and N7. Therefore, protonation of the N1 atom is expected to have a direct influence on the two highest energy $n\pi^*$ electronic transitions. The $\pi\pi^*$ transition energies are also expected to be affected because of overall change in electron density over the aromatic ring, but the effect is usually much smaller compared to the former.

Recently, we reported the SRCD and absorption spectra of a few selected adenosine mononucleotides at different pH of the medium [19]. Observing the pH sensitivities of band positions in those spectra we proposed in agreement with some earlier suggestions that most of the CD active transitions are $n\pi^*$ in nature [24], although the $\pi\pi^*$ transitions dominate the absorption spectra. Since the absorption spectra of LNA-A and β-Ado are practically similar, we label the pH sensitive CD bands of LNA-A also as mostly $n\pi^*$ type transitions. Along this line, a comparison between the CD and electronic absorption spectra of LNA-A reveals that the peak positions do not match. The transitions of the CD active bands appear to be hidden in the broad envelopes of the strong $\pi\pi^*$ absorption bands, and the mismatch is more for the bands in shorter wavelengths.

The spectrum of LNA-A at pH 10 is similar to that at pH 7 except for a decrease in the band intensities below 230 nm. Titration back and forth in pH reproduces the spectra, which implies no irreversible chemical modifications at low and high pH.

3.3. SRCD spectra of thymine nucleosides

SRCD spectra of the two anomers of thymidine, α-dThd and β-dThd, at pH 6 (nucleobase unionized) are presented in Fig. 5. We note that the spectrum of β-dThd is in fine agreement with that previously published by Sprecher and Johnson [28]. The spectra of α-dThd and β-dThd are nearly mirror images of each other, except for a small blueshift of the 272-nm band and a small redshift of the 192-nm band by 1 nm (β → α), which implies that the sign of the signal is determined by the configuration at C1′ of the sugar group to which thymine is linked. The rotational strengths are, however, different: the CD signal of α-dThd is larger than that of β-dThd at high wavelengths whereas the opposite is true at low wavelengths. The clear inversion of the signs of the CD bands by inversion of the

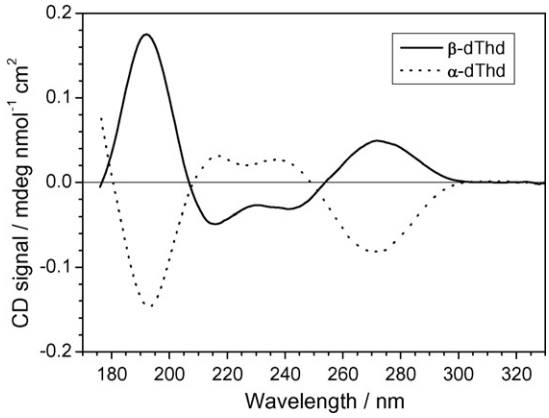


Fig. 5. SRCD spectra of α-dThd and β-dThd at pH 6.

configuration at C1′ for thymine nucleosides is different from previous observations for nucleosides of adenine. A comprehensive study by Ingwall [25] on the α and β anomers of the four D-pentofuranosides of adenine revealed that the oppositely signed CD was observed not for anomeric pairs but for those nucleosides that are enantiomer-like at C1′, C2′ and C3′. This is most likely due to steric effects since the presence of hydroxyl at C2′ in adenosine influences the sugar-base torsional angle that strongly determines the rotational strength [21,23]. In accordance with this interpretation, Miles et al. [21a] have observed that the pair of anomeric 6-methyl-2′-deoxycytidines give near-mirror-image circular dichroism curves in the range from about 210 to 300 nm.

The SRCD spectrum of LNA-T in the aqueous solution of pH 6 in the same spectral range (170–330 nm) is presented in Fig. 6, and to show the effect of methylene locking in the sugar moiety of the molecule, the CD spectrum of the thymine riboside (β-Thd) reported by Sprecher and Johnson [28] is included in the figure. Unlike LNA-A and β-Ado, the longest wavelength broad CD bands of LNA-T and β-Thd are positive and the band maximum appears at 270 nm. The intensity of this band in the case of LNA-T is nearly three times larger than that of β-Thd. In contrast, the positive band at 194 nm of β-Thd is about three

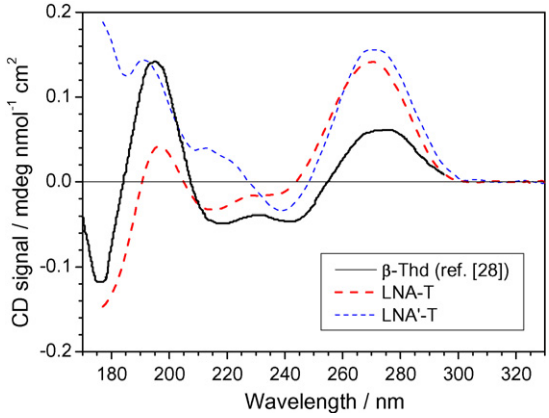


Fig. 6. SRCD spectra of LNA-T and LNA′-T at pH 6. The spectrum of Thd is reproduced from Ref. [28] (the Δε values in the original paper were multiplied by 0.033 to convert to the units used here).

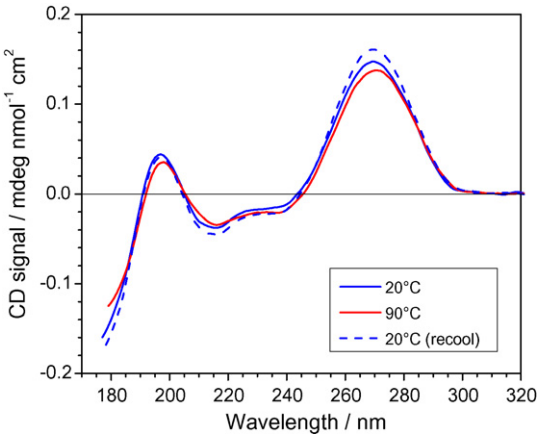


Fig. 7. SRCD spectra of LNA-T at 20, 90, and 20 °C after recooling.

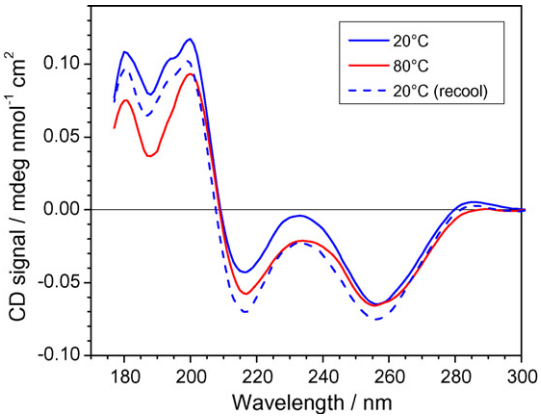


Fig. 8. SRCD spectra of LNA-A at 20, 80, and 20 °C after recooling.

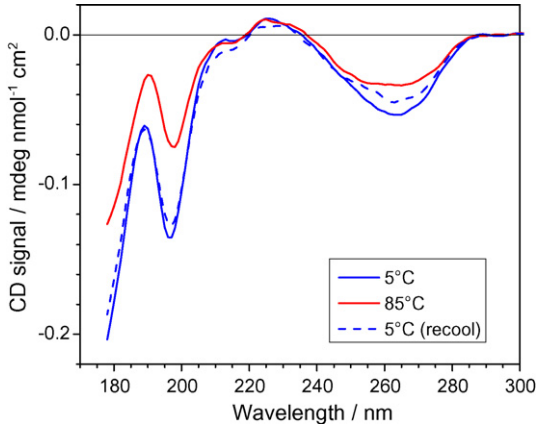


Fig. 9. SRCD spectra of β-Ado at 5, 85, and 5 °C after recooling.

times stronger than the 197-nm band of LNA-T. At 175 nm, LNA-T displays a very intense negative band similar to that of the unlocked molecule. Also, the features of the two weaker negative bands that have maxima at 215 and 239 nm appear practically similar in the two spectra. The apparent small shifts of their maxima are possibly due to stronger bands in the vicinity.

Another locked form of Thd was studied, LNA′-T, in which the configuration at C2′ differs in the oxygen-methylene linker pointing up instead of down (cf., Scheme 1). Its spectrum is similar to those of β-Thd and LNA-T above 230 nm (Fig. 6). However, the signal at 215 nm is positive in contrast to that of the other two. LNA′-T has a positive band at 192 nm in fair agreement with that of β-Thd, but displays a strong positive band with maximum below 176 nm. The data indicate that the sign of the 215- and 175-nm bands depends on the configuration at C2′ (in addition to C1′), likely due to steric hindrance in the rotation of the base relative to the sugar group about the glycosidic bond, as was discussed above.

Taken together, these results also clearly demonstrate that the nonchromophoric sugar and its configuration largely determines the CD spectrum of nucleosides in the VUV region.

3.4. Temperature variation

Temperature scans on solutions of locked nucleosides from 20 to 80–90 °C and back again to 20 °C reveal that the intensities of the CD bands change with temperature but no new spectral features develop (Figs. 7 and 8). In the case of LNA-T, the signal is about 10% higher after cooling to 20 °C from 90 °C but otherwise identical to the original spectrum at 20 °C (Fig. 7). The increased signal is most likely due to evaporation of water from the cell, and we conclude that LNA-T is thermally stable. Heating of LNA-A to 80 °C results in a decrease of the intensities of the bands in the VUV and an increase in the UV. After recooling to 20 °C some intensity is recovered in the VUV whereas the signal in the UV has decreased further. LNA-A is therefore somewhat thermally unstable.

We have performed similar experiments on β-Ado and find that the rotational strengths are reduced upon heating, but the SRCD bands are mostly reversible: there is a loss of 10–15% of the signal by heating the sample from 5 to 85 °C and cooling back again to 5 °C (Fig. 9). The ribose sugar moiety of the unlocked nucleoside exists in two pseudorotameric forms; C2′-endo (S-type) and C3′-endo (N-type) (Scheme 2) [39]. Quantum mechanical calculations predict that the relative stability of these two forms is nearly the same, and that they are separated by a low energy barrier (∼2 kcal/mol) [40]. Therefore, in aqueous solution in the temperature range of our measurement the two forms are always present in equilibrium mixture. Furthermore, there are suggestions that the rotational strengths depend on the relative values of the torsional angles between the sugar and base planes [21,23,26]. The conformational distribution among the isomers, which results due to this torsional motion, increases with temperature. All these factors contribute to lowering of rotational strength of the adenosine solution at higher temperature. The observed regaining of most of the original rotational strength at low temperature of adenosine indicates that energy barriers for pseudorotation of the sugar moiety and internal rotation between the sugar and base rings are low.

4. Conclusion

SRCD experiments on nucleosides and locked nucleosides reveal that the configuration of atoms in the sugar moiety

immensely influences the spectra, both in the position of the CD bands, their intensities and signs, and that the differences are largest in the VUV region. This study supports the interpretation that the CD bands are mainly due to $n\pi^*$ transitions in the nucleobase, based on a comparison between the absorption and CD spectra and the spectral changes upon adenine protonation. The origin of several bands has been pinpointed from comparisons of spectra of nucleosides differing in the configurations at chiral carbons in the sugar moiety. Thus, the configuration at C1′ (α and β anomers) determines the sign of all CD bands between 175 and 300 nm and that of C2′ the two bands at 175 and 215 nm in the case of thymidine nucleosides.

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