

Binding of an Acetic Acid Ligand to Adenosine: A Low-Temperature NMR Study

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Abstract: Binding of an acetic acid (HAc) ligand to adenosine (A) was studied by ¹H NMR spectroscopic techniques. Using a low-melting deuterated Freon mixture as solvent, liquid-state measurements could be performed in the slow exchange regime and allowed a detailed characterization of the formed associates. Thus, at 128 K, trimolecular complexes A·HAc₂ and A₂·HAc with both Watson–Crick and Hoogsteen sites of the central adenine base occupied coexist in various amounts depending on the adenosine:acetic acid molar ratio. Whereas the carboxylic acid OH proton is located closer to the acid for all hydrogen bonds formed, a more deshielded proton at the Watson–Crick site is evidence for a stronger hydrogen bond as compared to the Hoogsteen interaction. For the binding of acetic acid to an adenosine–thymidine base pair in either a Watson–Crick or a Hoogsteen configuration, hydrogen bonds to the available adenine binding site are strengthened as compared to the corresponding hydrogen bonds in the A·HAc₂ complex.

Introduction

The potential of nucleobases to form well-defined hydrogen-bonded base pairs is not only a major determinant of nucleic acid structure but is also fundamental to important biological processes such as replication and transcription. In addition, hydrogen-bond interactions between nucleobases and amino acid side chains are believed to play a crucial role in the recognition of specific nucleotide sequences by DNA-binding proteins.^{1a,b} Thus, whereas Watson–Crick pairing between the adenine (A) and the uracil (U) or thymine (T) base constitutes a structural key element in double-stranded RNA or DNA, multiple proton donor and acceptor sites at the adenine base enable its interaction with suitable functional groups in various geometries. Clearly, understanding the structural basis of ligand binding to adenine is not only important to DNA–protein interactions but also to the development of synthetic receptors for adenine derivatives.^{2a–i}

Acetic acid, a simple carboxylic acid, can not only serve as a bidentate adenine receptor molecule by use of its carboxylic acid functionality, but also as a model for aspartic or glutamic acid side chains involved in DNA–protein contacts. The

strength and specificity of adenine–carboxylic acid interactions has previously been investigated in aprotic solvents by NMR spectroscopic techniques.^{3–5} However, due to the fast exchange of individual species under the solution conditions employed, these studies failed to provide unambiguous information on the specificity and strength of individual hydrogen bonds formed. In particular, the preference of ligands to bind in either a Hoogsteen or a Watson–Crick geometry remains elusive. To circumvent averaging effects and to characterize hydrogen-bonded complexes by liquid state NMR in more detail, we have recently performed NMR measurements at very low temperatures by employing a deuterated Freon mixture CDCIF₂/CDF₃ as solvent. With measurements in the liquid state as low as 113 K, individual hydrogen-bonded complexes of nucleobases in slow exchange could be observed and characterized in detail for the first time in solution.^{6a–d} We now present NMR studies on the association of adenosine with acetic acid at low and ambient temperatures to explore the strength and preference of hydrogen bonding in the formed complexes.

Experimental Section

Methods. NMR experiments were performed on a Bruker AMX500 spectrometer. Temperatures were adjusted by a Eurotherm Variable Temperature Unit to an accuracy of ±1.0 °C. ¹H chemical shifts in chloroform at 293 K were referenced relative to CHCl₃ (δ_H = 7.24 ppm) and in a Freon mixture relative to CHClF₂ (δ_H = 7.13 ppm).

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Concentration-dependent chemical shifts were fitted with an appropriate equation by employing the Marquardt–Levenberg algorithm. Ab initio calculations were performed using SPARTAN, Version 4.1.1.

Materials. Reagents of the highest quality available were purchased from Sigma-Aldrich, Deisenhofen, Germany. $^{15}\text{NH}_4\text{Cl}$ was purchased either from Chemotrade, Leipzig (95% of label), or from Deutero GmbH, Kastellaun (99% of label). All reactions were controlled by TLC on silica gel plates (Merck silica gel 60 F₂₅₄). If necessary, solvents were dried by standard procedures prior to use. The deuterated Freon mixture $\text{CDCIF}_2/\text{CDF}_3$ was prepared as described⁷ and handled on a vacuum line which was also used for the sample preparation. 3',5'-Diacetyl-3- ^{15}N -2'-deoxythymidine was prepared from unlabeled 2'-deoxythymidine in analogy to procedures published previously.^{6b,8} 3',5'-Di-*O*-(triisopropylsilyl)-7- ^{15}N -2'-deoxyadenosine was synthesized according to literature procedures^{9,10} as described.^{6c}

The synthesis of 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-1- ^{15}N -adenosine was performed with some modifications based on a published procedure.¹¹

(a) 6- ^{15}N -Adenosine. After the addition of 0.38 g (6.97 mmol) of $^{15}\text{NH}_4\text{Cl}$ and 1.05 g (10.48 mmol) of KHCO_3 to 1 g (3.49 mmol) of 6-chloroadenosine dissolved in 5 mL of dimethyl sulfoxide, the reaction mixture was left for 3 days at 80 °C. It was subsequently diluted with 8.8 mL of distilled water, and the pH was adjusted to 7 using concentrated acetic acid. The solution was evaporated to dryness, and the product was isolated via column chromatography (SiO_2 , ethyl acetate–methanol 1:1). The purification afforded 750 mg (2.79 mmol, 80%) of 6- ^{15}N -adenosine. ^1H NMR (500 MHz, DMSO): δ (ppm) 3.54 (d, 1H, H5'), 3.65 (d, 1H, H5''), 3.98 (m, 1H, H4'), 4.15 (m, 2H, OH, H3'), 4.59 (m, 2H, OH, H2'), 5.88 (d, 1H, H1'), 7.39 (d, 2H, $^{15}\text{NH}_2$), 8.13 (s, 1H, H8), 8.37 (s, 1H, H2).

(b) 2',3',5'-Tri-*O*-(*tert*-butyldimethylsilyl)-6- ^{15}N -adenosine. To a solution of 0.75 g of 6- ^{15}N -adenosine (2.8 mmol) and 1.68 g of imidazole (24.64 mmol) in 2.8 mL of dry dimethylformamide (DMF) was added 1.86 g (12.32 mmol) of *tert*-butyldimethylsilylchlorosilane. The solution was stirred at room temperature for 20 h under an Ar atmosphere while being followed by TLC on silica gel plates. After removal of the solvent under reduced pressure, the syrup-like residue was dissolved in 50 mL of dichloromethane and mixed with 75 mL of 6% aqueous sodium hydrogen carbonate. The aqueous phase was extracted with 230-mL portions of CH_2Cl_2 . The combined organic phase was dried over sodium hydrogen carbonate, filtered, and concentrated by rotary evaporation to yield a gummy product. This product was purified by column chromatography (silica gel) using dichloromethane–methanol (10:0.2) as eluent to provide 1.11 g (1.82 mmol, 65%) of crystalline 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-6- ^{15}N -adenosine. ^1H NMR (250 MHz, CD_2Cl_2): δ (ppm) -0.043 (s, 3H, CH_3Si), -0.024 (s, 3H, CH_3Si), 0.14 (s, 3H, CH_3Si), 0.13 (s, 3H, CH_3Si), 0.12 (s, 3H, CH_3Si), 0.11 (s, 3H, CH_3Si), 0.78 (s, 9H, $(\text{CH}_3)_3\text{C}$), 0.94 (d, 9H, $(\text{CH}_3)_3\text{C}$), 0.95 (d, 9H, $(\text{CH}_3)_3\text{C}$), 3.79 (dd 1H, H5'), 4.01 (dd, 1H, H5''), 4.11 (q, 1H, H4'), 4.33 (t, 1H, H3'), 4.67 (t, 1H, H2'), 5.69 (d, $^{15}\text{NH}_2$), 5.99 (d, 1H, H1'), 8.15 (s, 1H, H8), 8.3 (s, 1H, H2).

(c) 2',3',5'-Tri-*O*-(*tert*-butyldimethylsilyl)-1-*N*-benzyl-6- ^{15}N -adenosine. To 1.09 g (1.786 mmol) of 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-6- ^{15}N -adenosine dissolved in 11 mL of dry DMF was added 0.42 mL (3.572 mmol) of freshly distilled benzylbromide. The reaction mixture was stirred at 40 °C for 2 days under an Ar atmosphere. The solvent was evaporated in vacuo, and the residue was purified by chromatography on silica gel using dichloromethane–methanol (10:

0.3) as eluent. The appropriate fractions were combined, and the solvent was removed to provide 0.91 g (1.166 mmol, 65.3%) of pure product. ^1H NMR (250 MHz, CDCl_3): δ (ppm) -0.043 (s, 3H, CH_3Si), -0.024 (s, 3H, CH_3Si), 0.14 (s, 3H, CH_3Si), 0.13 (s, 3H, CH_3Si), 0.12 (s, 3H, CH_3Si), 0.11 (s, 3H, CH_3Si), 0.78 (s, 9H, $(\text{CH}_3)_3\text{C}$), 0.94 (d, 9H, $(\text{CH}_3)_3\text{C}$), 0.95 (d, 9H, $(\text{CH}_3)_3\text{C}$), 3.72 (dd 1H, H5'), 3.91 (dd, 1H, H5''), 4.08 (q, 1H, H4'), 4.25 (t, 1H, H3'), 4.48 (t, 1H, H2'), 5.29 (d, 2H, CH_2Ph), 5.85 (d, 1H, H1'), 7.30 (m, 5H, Ph), 7.69 (s, 1H, H8), 7.90 (s, 1H, H2).

(d) 2',3',5'-Tri-*O*-(*tert*-butyldimethylsilyl)-6-*N*-benzyl-1- ^{15}N -adenosine. Addition of 63 mL of methanolic dimethylamine (1:1) to 0.96 g (1.230 mmol) of 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-1-benzyl-6- ^{15}N -adenosine resulted in a two-phase system, which was vigorously stirred at room temperature for 65 h. The reaction was followed by TLC on silica gel plates. After completion, the solvent was evaporated, and the dry residue was purified by chromatography on silica gel using dichloromethane–methanol (10:0.2) as eluent. The appropriate fractions were combined to provide after evaporation of the solvent 0.81 g (1.156 mmol, 94%) of pure 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-6-*N*-benzyl-1- ^{15}N -adenosine. ^1H NMR (250 MHz, CDCl_3): δ (ppm) -0.043 (s, 3H, CH_3Si), -0.024 (s, 3H, CH_3Si), 0.14 (s, 3H, CH_3Si), 0.13 (s, 3H, CH_3Si), 0.12 (s, 3H, CH_3Si), 0.11 (s, 3H, CH_3Si), 0.78 (s, 9H, $(\text{CH}_3)_3\text{C}$), 0.94 (d, 9H, $(\text{CH}_3)_3\text{C}$), 0.95 (d, 9H, $(\text{CH}_3)_3\text{C}$), 3.75 (dd 1H, H5'), 4.01 (dd, 1H, H5''), 4.11 (q, 1H, H4'), 4.32 (t, 1H, H3'), 4.70 (t, 1H, H2'), 4.87 (d, 2H, CH_2Ph), 5.99 (d, 1H, H1'), 6.10 (t, br, NH), 7.32 (m, 5H, phenyl), 8.05 (s, 1H, H8), 8.37 (d, 1H, H2).

(e) 2',3',5'-Tri-*O*-(*tert*-butyldimethylsilyl)-1- ^{15}N -adenosine. To 0.81 g (1.156 mmol) of 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-6-*N*-benzyl-1- ^{15}N -adenosine dissolved in a mixture of 8 mL of dichloromethane, 8 mL of acetonitrile, and 12 mL of distilled water were added 1 g (4.74 mmol) of NaO_4 and 6.77 mg (0.05 mmol) of $\text{RuO}_2 \cdot x\text{H}_2\text{O}$. The dark colored solution was stirred for 23 h at room temperature and filtered. The filtrate was treated with 50 mL of 6% aqueous sodium hydrogen carbonate and extracted with three 100 mL portions of dichloromethane. The organic extracts were combined, concentrated, and treated with 11.6 mL of methanol saturated with ammonia for 37 h. Subsequently, the solvent was removed and the dry residue was purified by chromatography on silica gel using dichloromethane–methanol (10:0.3). The appropriate fractions were pooled and evaporated to dryness to provide 0.545 g (0.894 mmol, 77%) of crystalline 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-1- ^{15}N -adenosine. $\text{M}(\text{C}_{28}\text{H}_{55}\text{N}_4^{15}\text{NO}_4\text{Si}_3) = 610$ g/mol. ^1H NMR (250 MHz, CD_2Cl_2): δ (ppm) -0.043 (s, 3H, CH_3Si), -0.024 (s, 3H, CH_3Si), 0.14 (s, 3H, CH_3Si), 0.13 (s, 3H, CH_3Si), 0.12 (s, 3H, CH_3Si), 0.11 (s, 3H, CH_3Si), 0.78 (s, 9H, $(\text{CH}_3)_3\text{C}$), 0.94 (d, 9H, $(\text{CH}_3)_3\text{C}$), 0.95 (d, 9H, $(\text{CH}_3)_3\text{C}$), 3.79 (dd 1H, H5'), 4.01 (dd, 1H, H5''), 4.11 (q, 1H, H4'), 4.33 (t, 1H, H2'), 4.67 (t, 1H, H2'), 5.73 (br, NH_2), 5.99 (d, 1H, H1'), 8.14 (s, 1H, H8), 8.28 (d, 1H, H2).

Results

Low-Temperature Measurements on Adenosine–Acetic Acid Association. One-dimensional ^1H NMR spectra showing the carboxylic acid OH and adenosine base proton resonances for a mixture of 3',5'-di-*O*-(triisopropylsilyl)-7- ^{15}N -2'-deoxyadenosine (^7A) and acetic acid (HAc) in a Freon solvent are plotted as a function of temperature in Figure 1. Upon cooling, the two amino proton signals (broadened at 273 K at around 7 ppm) shift downfield as a result of their increased participation in hydrogen-bond formation. A significant downfield shift is also observed for the adenine H8 proton, whereas adenine H2 is slightly upfield shifted at decreasing temperatures. H8 and H2 protons are easily assigned at higher temperatures because H8 is split into a doublet due to its scalar coupling $^2J_{\text{NH}} = 11.2$ Hz with the labeled endocyclic nitrogen-7 of ^7A . No resonances of carboxylic acid OH protons are observed above 193 K, but

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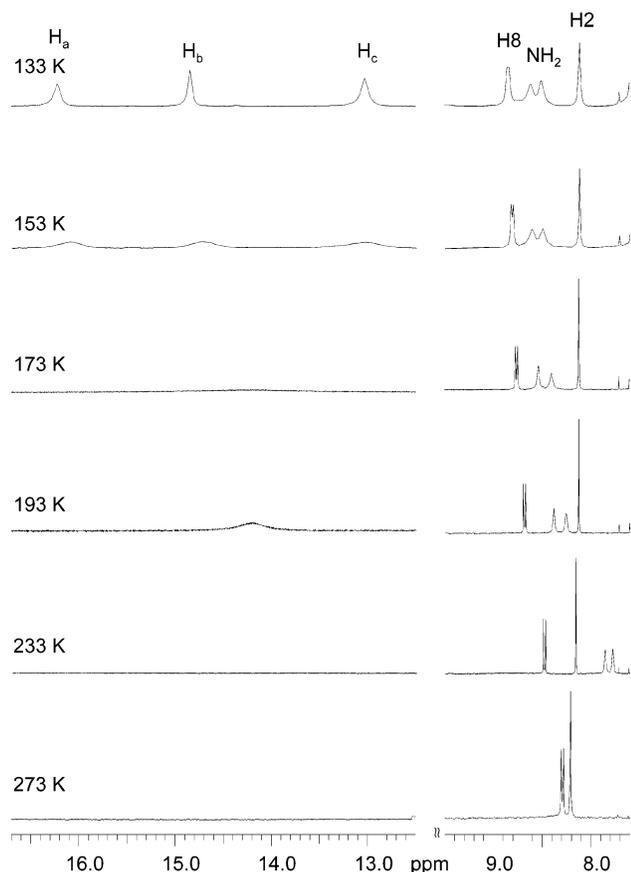


Figure 1. Temperature-dependent ^1H NMR spectra for a mixture of 3',5'-di-*O*-(triisopropylsilyl)-7- ^{15}N -2'-deoxyadenosine and acetic acid in Freon showing the OH (left) and base proton (right) spectral region.

they appear at lower temperatures. At 133 K, three carboxylic acid OH protons in slow exchange, H_a , H_b , and H_c , resonate at chemical shifts between 16.3 and 13 ppm. Due to their different solubility in Freon at very low temperatures, relative concentrations of A and acetic acid were reexamined by signal integration, yielding an adenosine:acetic acid molar ratio of 1:4 at $T \leq 133$ K.

^1H – ^1H NOE experiments under slow exchange conditions at 128 K provide a wealth of information about nucleoside conformation and A–HAc complex geometries (Figure 2). Thus, an intramolecular cross-peak observed between adenine H8 and the H2' sugar proton is stronger when compared to the cross-peak between H8 and H3' (spectral region not shown). Moreover, a cross-peak observed for H8 but missing for H2 with the 5'-triisopropylsilyl (TIPS) protecting group clearly establishes an anti orientation of the base relative to the sugar moiety. Consequently, the TIPS-protected 2'-deoxyadenosine adopts a C2'-endo sugar pucker with an anti glycosidic torsion angle.¹²

Likewise, carboxylic acid OH protons are easily assigned to different binding modes through ^1H – ^1H NOE contacts. As shown in Figure 2, the two OH protons at 14.90 ppm (H_b) and 16.30 ppm (H_a) having equal intensity show NOE contacts to adenosine H8 and H2 protons, respectively. Clearly, H_b and H_a are associated with Hoogsteen and Watson–Crick bound acetic acid molecules. The Hoogsteen H_b signal also shows NOE

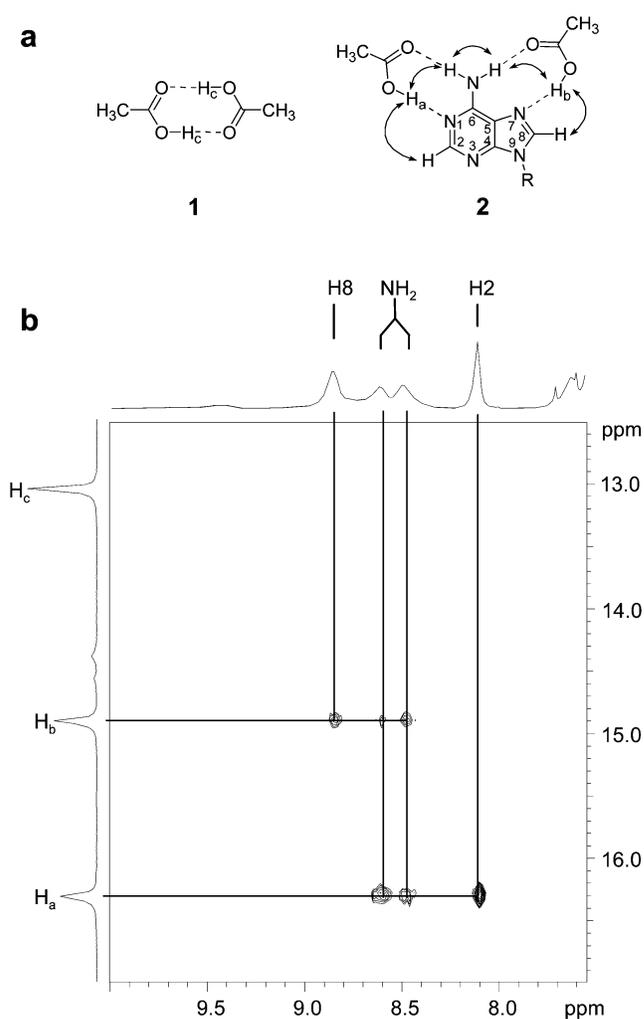


Figure 2. (a) Geometries of acetic acid dimer **1** and A·HAc₂ complex **2** with arrows indicating NOE contacts observed in a Freon solution at 128 K. (b) Portion of a 2D NOE spectrum of 3',5'-di-*O*-(triisopropylsilyl)-7- ^{15}N -2'-deoxyadenosine and acetic acid (1:4) in Freon showing cross-peaks between OH and adenine base protons. The connectivities are indicated by solid lines. The spectrum was acquired at 128 K with an 80 ms mixing time.

contacts to the adenosine 5'-TIPS protecting group, further substantiating its binding at the Hoogsteen site of A with anti conformation (not shown). In addition, cross-peaks of H_a and H_b to the same two amino protons with reversed relative intensity indicate their binding to the same adenine base in a single $^7\text{A}\cdot\text{HAc}_2$ complex **2** (Figure 2). No additional sets of resonances are seen for adenine H2, H8, and amino protons, confirming the presence of adenosine in a single 1:2 complex. The remaining H_c proton resonance at 13.04 ppm exhibits no NOE contact to adenine base protons and can be unambiguously assigned to an acetic acid dimer **1** by comparison with a low-temperature measurement on a pure acetic acid solution.

We could not detect any resolved scalar coupling of the Hoogsteen bound H_b resonance to the labeled adenine N-7, indicating localization of the proton closer to the oxygen of acetic acid in a neutral complex. To explore the possibility of trans-hydrogen scalar coupling in the case of the Watson–Crick bound proton and N1 of adenosine, 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)-adenosine was selectively ^{15}N -labeled at N-1 and used for the association studies with acetic acid in Freon. *tert*-

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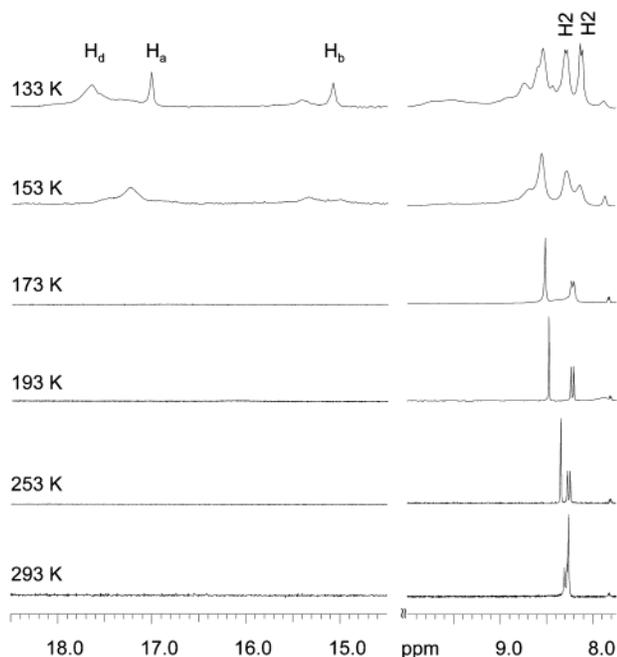


Figure 3. Temperature-dependent ^1H NMR spectra showing the OH and base proton spectral region for a mixture of 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)- $1\text{-}^{15}\text{N}$ -adenosine and acetic acid (1:0.7) in Freon.

Butyldimethylsilyl (TBDMS)-protected adenosine (^1A) has been found to possess favorable solubility properties in Freon especially at lower temperatures, while at the same time NOE contacts between H8 and H2' (strong) and the 5'-TBDMS protecting group show that it adopts an anti conformation like the TIPS-protected deoxyribonucleoside. In Figure 3, the OH and base proton spectral regions for a mixture of $1\text{-}^{15}\text{N}$ -adenosine and acetic acid with a molar ratio $^1\text{A}:\text{HAc}$ of >1 in Freon are plotted as a function of temperature. Due to the ^{15}N label at position 1 of the adenine base, H2 protons are easily assigned on the basis of their scalar coupling of $^2J_{\text{NH}} = 15.6$ Hz to N1. Again, a downfield shift is observed for the adenine H8 proton, whereas adenine H2 moves upfield upon lowering the temperature. However, in contrast to the $^7\text{A}-\text{HAc}$ mixture, both H2 and H8 resonances of ^1A split into at least two different signals below 173 K. In particular, two H2 doublets at 8.11 and 8.27 ppm are clearly identified in the 1D spectra (see Figure 3), while two different H8 resonances with chemical shifts of 8.53 and 8.60 ppm can be assigned by their corresponding NOE contacts to the sugar protons in a 2D NOE spectrum at 128 K (not shown). It has to be noted that at this temperature the stoichiometry of adenosine and acetic acid was found to be about 1:0.7 based on signal integration.

Again, the acid OH signals are only observed at low temperatures. At 153 K, the OH resonances are considerably broadened and finally split into three predominant signals, H_d , H_a , and H_b , at 133 K with chemical shifts of $\delta = 17.76$ ppm, $\delta = 17.10$ ppm, and $\delta = 15.11$ ppm. Obviously, these resonances must be attributed to different coexisting species in slow exchange, and their assignment makes use again of 2D NOE experiments under slow exchange conditions at 128 K. As seen from the 2D NOE spectrum in Figure 4a, H_a and H_b resonances are easily assigned to Watson-Crick and Hoogsteen bound acetic acid in a $^1\text{A}\cdot\text{HAc}_2$ complex **2** based on cross-peaks to adenine H2, H8, and the same two amino protons (vide

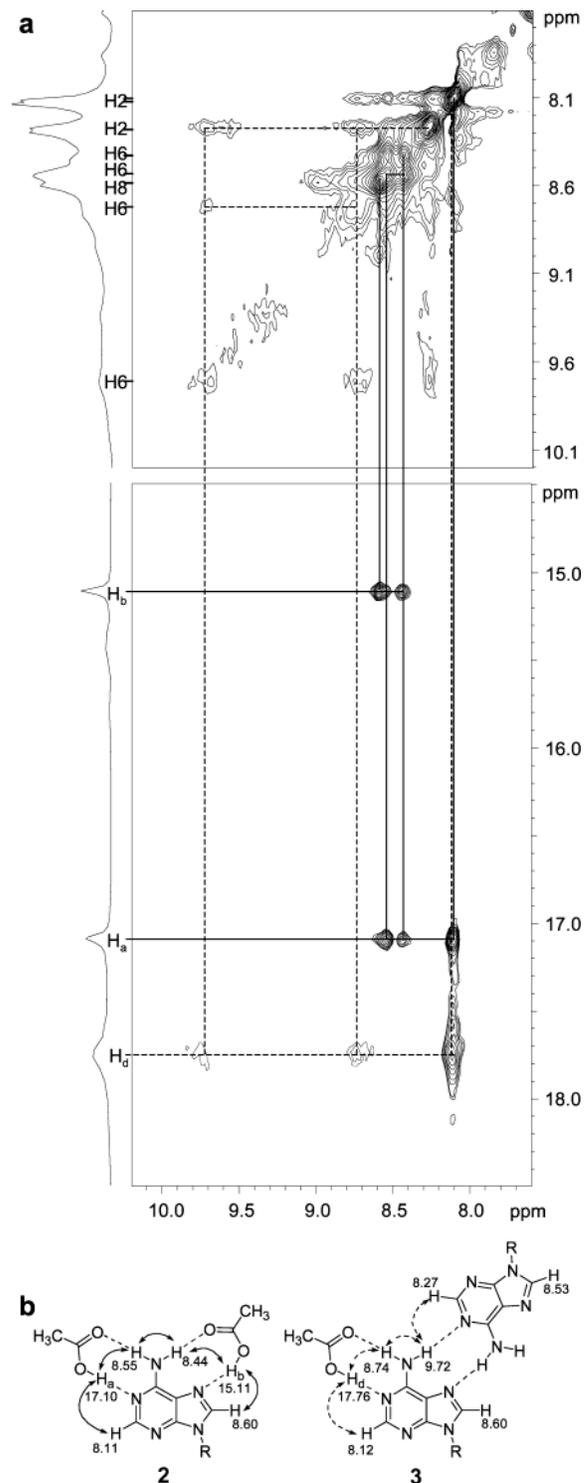


Figure 4. (a) Portion of a 2D NOE spectrum of 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)- $1\text{-}^{15}\text{N}$ -adenosine and acetic acid (1:0.7) acquired in Freon at 128 K with an 80 ms mixing time showing base-base (top) and OH-base cross-peaks (bottom). The connectivities are indicated by solid and dashed lines. (b) Geometries of complexes **2** and **3** formed between **A** and **HAc** with arrows indicating NOE contacts observed in a Freon solution at 128 K. Also given are ^1H chemical shifts δ of base and acid OH protons.

supra). Again, no resolvable scalar coupling between H_a and the labeled nitrogen-1 is observed, in agreement with localization of the proton closer to the oxygen of acetic acid. The additional broad and most downfield shifted H_d signal at 17.76 ppm exhibits cross-peaks with an adenine H2 at 8.12 ppm, slightly

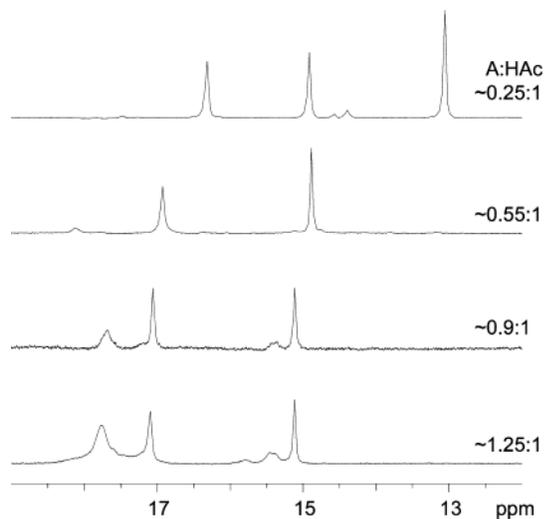


Figure 5. Dependence of ^1H NMR spectra on the adenosine:acetic acid molar ratio in a Freon solvent at 123 K.

downfield shifted with respect to H2 in **2** at 8.11 ppm (Figure 4a). Moreover, weak but noticeable cross-peaks are observed between H_d and amino protons at 8.74 ppm (stronger, i.e., hydrogen bonded to acetic acid) and 9.72 ppm (weak). These in turn have NOE contacts to the H2 proton at 8.27 ppm, which has no additional contacts to OH acetic acid protons. Taken together, this pattern of NOE connectivities suggests the formation of a 2:1 $^1\text{A}_2\cdot\text{HAc}$ complex **3** with acetic acid bound at the Watson–Crick site and a second adenine base at the Hoogsteen site.

The analysis of signal intensities not only supports the coexistence of complexes **3** and **2** but also indicates a molar ratio **3:2** of about 2:1: (i) with an approximate stoichiometry of adenosine and acetic acid of 1:0.7, relative signal intensities for H_d , H_a , and H_b are found to be 2:1:1; (ii) the relative intensities of the H2 proton signals at 8.27 ppm and 8.11/8.12 ppm are about 2:3; and (iii) coexistence of **2** and **3** accounts for one H8 resonance of the Hoogsteen bound adenosine in **3** at 8.53 ppm and for the two other H8 signals being isochronous at 8.60 ppm.

Moreover, on the basis of its downfield chemical shift, the amino proton with $\delta = 9.72$ ppm is expected to be involved in a NHN rather than a NHO hydrogen bridge as inferred from homoassociation studies of adenosine and 6-methyl-adenosine (K. Weisz, unpublished results). It should also be mentioned that OH protons of acetic acid show weak NOE contacts to their own methyl group (not shown). The methyl resonance is split into at least 2–3 components for all stoichiometries, reflecting the different environment of the acetic acid molecule in the complexes.

^1H NMR spectra showing the OH proton region of adenosine–acetic acid complexes are plotted in Figure 5 as a function of the A:HAc molar ratio. With acetic acid present in excess, Watson–Crick and Hoogsteen sites of adenine are completely occupied by acid molecules, giving rise to $\text{A}\cdot\text{HAc}_2$ complexes **2** and additional formation of acetic acid dimers **1** having a more upfield shifted OH resonance (Figure 5, top). Upon a lowering of the acid concentration, acetic acid dimers disappear at stoichiometric ratios of A:HAc ~ 0.5 and complexes **2** coexist with a new trimeric $\text{A}_2\cdot\text{HAc}$ complex **3** that is increasingly formed at ratios > 0.5 and characterized by a broad downfield

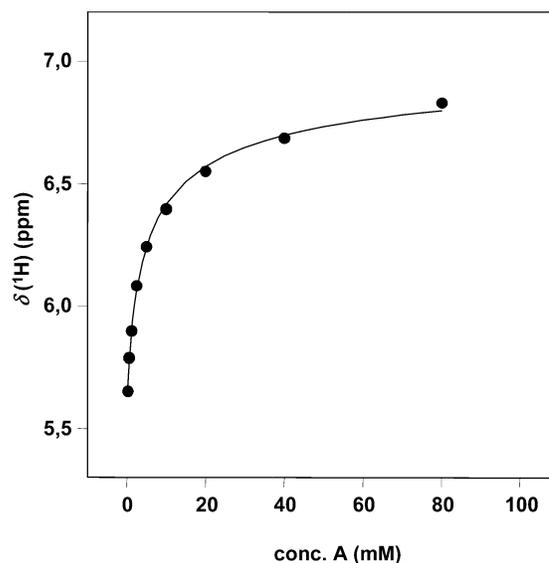


Figure 6. Concentration-dependent adenine amino proton chemical shift in a 1:1 mixture of 3',5'-di-*O*-(triisopropylsilyl)-2'-deoxyadenosine and acetic acid in CDCl_3 at 293 K. The line represents the least-squares fit.

shifted OH resonance at about 17.8 ppm (Figure 5, bottom). No effort was made to assign additional OH proton signals of minor species due to their low intensity. Note that differences observed in OH chemical shifts for solutions of different molar ratios and especially apparent for the Watson–Crick bound OH signal in the top spectrum arise from Freon mixtures of slightly different composition. Because the proton transfer in a neutral hydrogen-bonded complex is associated with a charge separation, the hydrogen-bond geometry and hence the proton chemical shift are very sensitive to the temperature- and composition-dependent dielectric constant ϵ of the Freonic solvent.^{13a,b}

Concentration-Dependent Measurements on Adenosine–Acetic Acid Association. Considering the higher-order complexes **2** and **3** formed at low temperatures, there seems to be no unambiguous and easy way to determine an association constant between adenosine and acetic acid. Surprisingly, however, a good fit of the concentration-dependent amino proton chemical shift is achieved by employing a simple 1:1 association model between 3',5'-di-*O*-(triisopropylsilyl)-2'-deoxyadenosine and acetic acid in chloroform at 293 K (Figure 6).

Due to the broadening of carboxylic acid OH resonances under these conditions, concentration-dependent chemical shifts of the adenine amino protons (in fast exchange) were used for the determination of the association constant. Values obtained from the least-squares fit amount to $296 \pm 43 \text{ M}^{-1}$ for the association constant as well as to 5.56 and 7.08 ppm for the limiting chemical shift of monomer and complex, respectively. With an association constant of 45 M^{-1} determined for adenine complexation with thymidine under the same experimental conditions (not shown), the $\text{A}\cdot\text{HAc}$ dimer is more stable as compared to the AT base pair. The good fit with a 1:1 model may be indicative of predominant formation of 1:1 complexes at ambient temperatures. Nevertheless, the determined association constant only represents an average over the various association modes, and the limiting chemical shift at infinite

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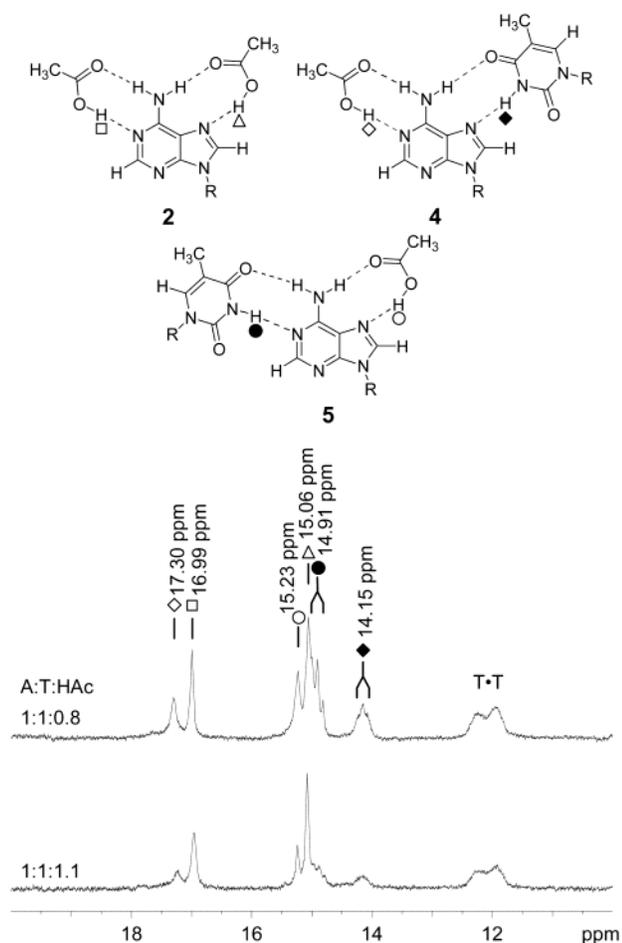


Figure 7. ^1H NMR spectra of a mixture of adenosine, $3\text{-}^{15}\text{N}$ -2'-deoxythymidine, and acetic acid in Freon at 133 K showing the acetic acid OH and thymidine imino proton spectral region with different acetic acid concentrations. The proton resonances are assigned to the complexes indicated on top.

concentration is actually an average over all coexisting species in solution. Comparing the value of 7.08 ppm to the value of 7.98 ppm, obtained in a separate titration experiment for adenosine homodimers, indicates that amino protons engaged in a $\text{N}-\text{H}\cdots\text{O}$ hydrogen bond with the acid are actually more upfield shifted as compared to amino protons in a $\text{N}-\text{H}\cdots\text{N}$ hydrogen bond formed in the adenosine self-associates.

Measurements on Ternary Mixtures of Adenosine, Thymidine, and Acetic Acid. Because in nucleic acid structures the adenine base is often hydrogen bonded to a thymine or uracil base to form Watson–Crick or in some cases Hoogsteen base pairs, we also examined the association in a ternary mixture of free adenosine, 2'-deoxythymidine, and acetic acid. To simplify the analysis of the spectra, we decided to employ specifically $3\text{-}^{15}\text{N}$ -labeled 2'-deoxythymidine (T), which provides for a convenient discrimination between thymine imino and acid OH protons (vide infra). As shown in Figure 7, the ^1H NMR spectrum of a 1:1:0.8 adenosine–thymidine–acetic acid mixture in Freon at 133 K displays several T imino and acid OH protons at low field. The T imino resonances at 14.91 and 14.15 ppm are easily identified by their one-bond scalar coupling to ^{15}N , giving rise to a doublet with $^1J_{\text{NH}}$ of about 90 Hz and a central resonance due to residual ^{14}N isotopomers ($\sim 37\%$). Also, imino

resonances characteristic of thymidine homodimers appear at around 12.1 ppm and are still exchange broadened at 133 K. The two OH resonances at 16.99 and 15.06 ppm of about equal intensity must be attributed to Watson–Crick and Hoogsteen bound acetic acid molecules in a ternary $\text{A}\cdot(\text{HAc})_2$ complex **2** (vide supra). Further information can be obtained from a 2D NOE spectrum at 133 K (not shown). At this temperature, very weak exchange cross-peaks are still observed between the two downfield shifted thymidine imino protons and the imino protons in the homodimers. As expected for the OH resonances in **2**, the OH signals at 16.99 and 15.06 ppm exhibit a NOE contact to adenine H2 and H8 (identified by a cross-peak to adenosine sugar protons), respectively. Moreover, the most downfield shifted OH proton at 17.30 ppm is connected to adenine H2 and thus Watson–Crick bound to the adenine base.

The assignment can be completed by further considering signal intensities. Thus, about equal intensities are found for the OH resonance at 17.30 ppm and the TNH signal at 14.15 ppm. The same applies to the OH and NH resonances at 15.23 and 14.91 ppm, respectively. Such a situation is compatible with the formation of two different ternary $\text{A}\cdot\text{T}\cdot\text{HAc}$ complexes **4** and **5** (Figure 7). Obviously, the former two protons are positioned in **4** with acetic acid bound in a Watson–Crick and thymidine bound in a Hoogsteen fashion. The latter two protons are associated with complex **5**, where HAc and T ligands have changed binding sites. Note that, in contrast to T homodimers, no acetic acid dimers are observed in the spectra. By increasing the acetic acid concentration, signal intensities of the T containing ternary complexes **4** and **5** decrease, and $\text{A}\cdot(\text{HAc})_2$ complexes **2** clearly predominate (Figure 7, bottom).

Discussion

Strength of Hydrogen Bonds in Adenosine–Acetic Acid Complexes. Having unambiguously assigned proton resonances to particular complex structures in slow exchange allows the extraction of important information regarding hydrogen-bond contacts. Thus, the proton chemical shift can be used as a sensitive indicator for the relative strength of hydrogen bonds. A more deshielded proton in the $\text{O}-\text{H}\cdots\text{N}$ hydrogen bridge originates from a displacement of the proton toward the acceptor atom and is associated with a shortening of the hydrogen bond.^{6c,14a,b} Experimentally, a stronger Watson–Crick $\text{O}-\text{H}\cdots\text{N1}$ hydrogen bond is clearly indicated by its more downfield shifted OH resonance. However, for all complexes observed, the acetic acid OH proton is still localized closer to the oxygen of acetic acid in a nonzwitterionic complex. Due to the absence of 1:1 complexes, experimental data related to the hydrogen-bond strength in $\text{A}\cdot\text{HAc}$ associates are only available for the simultaneous binding of two carboxylic acid ligands in a ternary complex **2**. Although low-level ab initio calculations point to a slight weakening of hydrogen bonds upon binding of a second carboxylic acid ligand, the relative strength of hydrogen bonds within a 1:2 complex should closely follow the situation in the corresponding 1:1 dimers.

Stronger acetic acid binding is observed when substituting one acetic acid molecule in the $\text{A}\cdot(\text{HAc})_2$ complex by a second adenine or a thymine as evidenced by a downfield shift of the

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OH resonance. Thus, a shorter hydrogen bond between acetic acid and A N1 or A N7 is observed when thymidine is simultaneously base-paired to A. Clearly, this situation bears special relevance when ligand binding to AT base pairs, for example, in nucleic acid structures, is of major interest.

Structure of Complexes. For the association of adenosine with acetic acid at low temperatures, trimolecular complexes are formed. Thus, in the presence of acetic acid, adenosine forms various amounts of 1:2 A·HAc₂ and 2:1 A₂·HAc complexes depending on the molar ratio of nucleoside to ligand. With an excess of acetic acid, both the Watson–Crick and the Hoogsteen binding site of the adenine base are occupied by acid. Upon an increase in the adenosine:acetic acid molar ratio to >0.5, increasing amounts of A₂·HAc complexes are formed at the expense of the 1:2 associates. It is interesting to note that even at a molar ratio of 1:1 no binary 1:1 adenosine–acetic acid complexes have been detected but rather 2:1 and 1:2 complexes coexist in equal amounts. Obviously, the enthalpy gain of such a situation (more hydrogen-bond interactions are formed) outweighs the entropic penalty in a trimolecular complex, at least at low temperatures.

In contrast to our findings, adenine binding studies with various carboxylic acids at ambient temperatures have mostly been interpreted in terms of bimolecular associates, although evidence for the formation of 1:2 adenine–acid complexes was provided at higher concentrations for some acids.^{5,15} Clearly, whereas lower temperatures will increasingly favor aggregation to larger clusters, 1:1 complexes are likely to predominate at higher temperatures especially at low concentrations. This is also indicated here by the concentration-dependent chemical shift data of adenine amino protons in the presence of an equimolar amount of acetic acid in chloroform at 293 K that fit to a simple 1:1 association model (Figure 6). The corresponding association constant of $K_{\text{ass}} = 296 \text{ M}^{-1}$ is in line with a $K_{\text{ass}} = 160 \text{ M}^{-1}$ that has been previously determined for 9-ethyladenine–butyric acid association in chloroform at 303 K.³

In general, both Watson–Crick and Hoogsteen binding modes have been reported in the past to be operative depending on the particular ligand. NMR studies on adenine binding sites either have employed NOE experiments or have followed the chemical shift of adenine H2 and H8 protons in the presence of the ligand. Thus, by adding butyric acid to 9-ethyladenine, a downfield shift of the adenine H8 resonance and an upfield shift of the H2 signal was observed by Lancelot.³ By taking downfield and upfield shifted H8 and H2 resonances as evidence for a Hoogsteen and Watson–Crick bound acid, respectively, aromatic carboxylic acids have indicated a distinct preference for the Hoogsteen site, whereas aliphatic acids were found to prefer the Watson–Crick site for binding adenine.⁵ Indeed, we observe corresponding upfield and downfield shifts of H2 and

H8 signals upon formation of the 1:2 complex **2** with Watson–Crick and Hoogsteen bound acid. When compared to free adenosine, differences in the ¹H chemical shift for H2 and H8 in **2** at low temperatures amount to about –0.2 and +0.8 ppm, respectively (see also Figure 1).

On the basis of the hydrogen-bond strength, Watson–Crick binding should be preferred by acetic acid, but solvation effects also contribute to the free enthalpy of association and hence to the extent of specific complex formation. Whereas at low temperatures no preferential adenine binding site for acetic acid can be assigned due to the lack of any 1:1 complex, the acid preferentially occupies the Watson–Crick site in the A₂·HAc complex **3**. However, a broad low-intensity peak downfield from the Hoogsteen OH resonance also points to a small population of Hoogsteen bound acid in such 2:1 associates (see Figure 5). Broadening of carboxylic acid protons in the latter complexes may be attributed to exchange processes, for example, rapid dissociation of the second weakly bound adenine base and possibly a concomitant exchange between its N1 and N7 atoms as a hydrogen-bond acceptor.

In addition to Watson–Crick and Hoogsteen binding, there is also the possibility of acetic acid binding to adenine N3 through only one hydrogen bond. This binding mode, although only populated to a small extent, was considered in adenine–carboxylic acid associations at ambient temperatures.^{3,4} However, on the basis of the present studies on adenosine–acetic acid mixtures, such a binding can be excluded.

Conclusion

The study of low-molecular weight host–guest interactions by NMR is often hampered by the fast exchange of species coexisting in solution. To overcome this problem, we have performed measurements at temperatures low enough to be in the slow exchange regime, thus allowing for the unambiguous characterization of the structures and interactions present. The results obtained here for the complexation of adenosine or adenosine–thymidine base pairs with acetic acid provide new insight into the strength and specificity of binding. Clearly, this information is not only of relevance to protein–nucleic acid interactions which may include acidic amino acid side chains of aspartic and glutamic acid. It is also important for the development of potent receptors for nucleic acid bases, which rely on the specificity and affinity for binding their target.

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