Base Pair Formation of Free Nucleobases and Mononucleosides in the Gas Phase

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Abstract: The native affinity of the nucleobases of DNA to form pairs is investigated by studying the gas phase behavior of clusters of free unsubstituted nucleobases and mononucleosides. The formation of neutral bimolecular clusters of these compounds in the gas phase has been studied by IR laser desorption of the neutral molecules into a supersonic beam expansion. Complementary nucleobase pairs as found in DNA, these being adenine–thymine and cytosine–guanine pairs, have been found to be formed in preference to noncomplementary base pairs. "Pseudo association constants" for the formation of the dimers of free nucleobases and nucleosides in the gas phase are calculated from the experimental results. A strong influence due to side groups affecting the dimer formation of the nucleobases is shown.

Introduction

The famous Watson–Crick structure of the double stranded helix of nucleic acids[1] now celebrates its 40th anniversary. The forces causing the double stranded structure of DNA are hydrogen bonding between complementary nucleobases of the two strands, hydrophobic forces formed by overlapping π-systems of nucleobases of the two strands, steric forces due to the rigid backbone—the sugar–phosphate chain—of the strands and interactions between phosphate ions of the DNA and solvent molecules. The specific formation of adenine (A) and thymine (T) pairs on the one hand and cytosine (C) and guanine (G) on the other, the base pairs exclusively occurring in DNA, may be a result of these forces or of specific affinities of the nucleobases themselves.

Our intention is to study unmodified nucleobases and the corresponding mononucleosides in the gas phase to investigate whether the complementary nucleobase pairs represent native affinities of the nucleobases and hence are already to be found in the gas phase or are mediated by other effects as the structure of the whole DNA. In particular we feel that it is important to study the nucleobase pair formation in the absence of any side groups, which may lead to false conclusions.

The complementary nucleobase pairing within the double helical structure of the DNA requires pairing of each adenine residue with a thymine residue and each cytosine residue with a guanine residue. Discussions about the formation of nucleobase pairs and the forces stabilizing them date back to the original discovery of the DNA structure. Theoretical approaches to structures and stabilizing energies of nucleobase pairs were made in several attempts to predict gas phase[2-6] as well as liquid phase[7-9] behavior. Due to the number of atoms of nucleobase dimers, however, simplifications of the calculations are required such as using minimal base sets, reduction of geometric influences, estimation of some input values or the use of empirical or semi empirical potentials. These simplifications, however, reduced the accuracy of most of the theoretical results for nucleobase pair formation.

The geometric compatability of the nucleobases in the double helical structure of the DNA was generally supposed to be the reason for exclusive formation of the Watson–Crick base pairs A-T and C-G. The experimental investigation of isolated nucleobases and their interactions, however, is of interest to prove this statement. Some nucleobase pair structures found in different environments are shown in Figure 1. Besides the hydrogen bonded Watson–Crick pairs (Figure 1, **1** and **2**) other hydrogen bonded arrangements of free nucleobases have been found. These are due to hydrogen bonds formed at other positions of the nucleobases. Cocrystallization of adenine and thymine may result in formation of A-T pairs in the Hoogsteen geometry[10] (Figure 1, **3**). However, not only hydrogen bonds may stabilize isolated nucleobase clusters, but van der Waals interactions may as well. Overlapping of the π-systems of nucleobases causes the formation of stacks of planar parallel nucleobases (Figure 1, **4**). However, van der Waals interactions between different nucleobases show no selectivity. Thus a predominate formation of complementary base pairs due to these forces could not be observed. Stacked base pairs have been found highly stabilized in aqueous solutions due to hydrophobic forces between the nucleobases.[11] The degree of formation of stacked base pairs in water however is mostly not due to interactions between the nucleobases but due to the influence of solvent molecules.[12,13] Hence it becomes of interest to see if selectivity is observable in the absence of the solvent.

Several experimental investigations of substituted nucleobase pairs in the gas phase[14] and in different solvents[15-18] have been performed. The proportion of substituted nucleobases and

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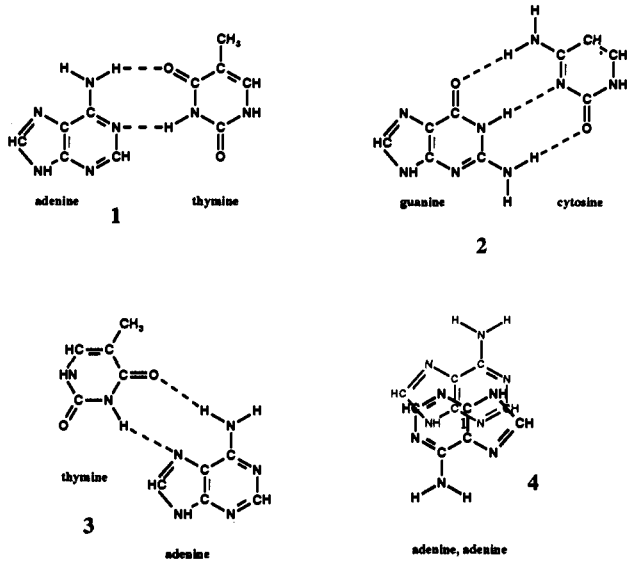

Figure 1. Some possible configurations of nucleobase pairs. The Watson–Crick configurations of A-T (**1**) and C-G (**2**) as found in DNA are hydrogen bonded. Another hydrogen bonded configuration shown for the A-T nucleobase pair is the Hoogsteen geometry (**3**). An example for base stacking is shown as a stacked A-A pair (**4**).

nucleobase dimers in solution was determined by IR spectroscopy[19,20] and NMR studies.[21,22] In general, experiments have shown that a hydrogen bonded configuration of nucleobase pairs is more favored for nonpolar solvents[23] while a stacked configuration is preferred in aqueous solutions.[24] These results demonstrate the strong influence of the solvent on the nucleobase pair formation behavior.

To examine the contribution of the nucleobases themselves on the forces that cause the formation of definite complementary base pairs, the interactions of free nucleobases in the gas phase are here investigated. We used nucleobases without substitution to exclude side group interactions within the formation of the nucleobase dimers. We felt that investigating the free nucleobases in the gas phase, the question could be solved whether the nucleobases themselves form A-T and C-G pairs or whether the formation of the Watson–Crick pairs is due to the three dimensional structure of the DNA, external effects, or a combination of both. The free unsubstituted bases were chosen to exclude any side group interactions as they will be shown to influence the nucleobase pair formation decisively. By using free nucleobases, any interaction can only take place between the nucleobases. Only measurements in the gas phase should give unequivocal information about nucleobase pairs formed by intermolecular forces between the nucleobases and not by solvent dependent interactions.

Investigating the cluster behavior of free nucleosides in the gas phase we found a change in the predominantly formed dimers of the nucleosides compared to the dimer formation of the nucleobases. This change is attributed to side group effects affecting the dimer formation of nucleobases. Nucleosides, such as adenosine (Ad), deoxythymidine (Td), cytosine (Cd), and guanosine (Gd) differ from the nucleobases by addition of a ribose molecule to the N^1 atom at the pyrimidine bases or to the N^9 atom at the purine bases.

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Experimental Section

Experiments were performed in a reflectron time of flight mass spectrometer (RETOF-MS) described in detail elsewhere;[25] therefore, only a brief description is presented here.

A pulsed CO_2 laser (Pulse System Inc.) was used for desorption of the sample molecules. The IR laser beam was slightly focused onto the solid sample through a 8 cm ZnSe lens. A typical desorption irradiation used was 10^4 W/cm^2 at 10.6 μm. The sample probe was positioned ca. 1 mm in front of a pulsed jet valve inside the desorption chamber. During a laser shot the pulsed valve was opened to emit a pulse of Ar atoms. The sample molecules were desorbed into a supersonic beam of Ar. The jet provides cooling of the internal degrees of freedom of the molecules due to multiple collisions. The formation of any neutral clusters takes place during these multiple collisions. Through a skimmer the neutral sample molecules and clusters were transported into the ion source of the RETOF-MS.

Ions, ionic fragments, and ionic clusters produced during the desorption into the jet were prevented from entering the ion source by a positive voltage on the source repeller plate. The neutral molecules and clusters entering the ion source were postionized with multiphoton ionization (MUPI). Pulsed radiation at variable wavelength was provided by the frequency doubled (BBO crystal) output of a excimer laser (XeCl, LPX100 Lambda Physik Göttingen) pumped dye laser (FL3002 Lambda Physik Göttingen). The UV laser beam was focused into the ion source by a 20 cm fused silica lens. A typical ionization wavelength used was 260 nm. This wavelength was chosen because all sample molecules absorbed at this wavelength and the photon energy was sufficient to perform two-photon ionization. The laser intensity could be adjusted up to 2×10^8 W/cm^2 by neutral density filters. The positive ions produced by MUPI post-ionization were separated and detected in the RETOF-MS with a typical fwhm resolution of ca. 3000 at mass 300. Each spectrum represents the sum of 25 spectra recorded using a 200 MHz transient digitizer (Le Croy).

Sample preparation was maintained to avoid any dimer formation of the sample molecules before desorption. The samples were used without further purification as provided by the manufacturer (Serva Chemicals, Heidelberg). Sample preparation was performed without any solvents or additional chemicals, but by mixing the samples in a mortar and compressing them into thin layers. The pure nucleobase or mononucleoside samples were introduced to the desorption chamber and analyzed in the manner described.

Results and Discussion

The mass spectra of the four nucleobases occurring in natural DNA were obtained, as laser desorption of intact neutral molecules into a supersonic beam in combination with MUPI provides a powerful tool to investigate biomolecules.[26] With this method, time of flight mass spectra of native samples could be observed without significant fragmentation. Desorption and ionization were adjusted to minimize fragmentation in both steps. The intensity of the ionizing laser was chosen to ensure that the neutral sample molecules would absorb only two photons and not three or more. The wavelength of the ionizing laser was chosen to allow significant ionization without fragmentation for all four nucleobases. At a wavelength of 260 nm all four nucleobases could be ionized by absorbing two photons. Therefore, two photon post-ionization was performed with 260 nm (4.77 eV) and 10^7 W/cm^2. The first photon excites the sample molecules into an intermediate state. Only a real intermediate state features the advantages of resonant enhanced ionization. Due to the size of the nucleobases however the density of states is high enough to provide at least one real intermediate state to be excited in each of the four nucleobases at 260 nm within the bandwidth of the ionizing laser. By absorbing a second photon the excited molecule will be ionized as the energy of two photons (9.54 eV) is higher than the ionization energies of the nucleobases (7.8–8.8 eV). The excess energy deposited in the molecular ions by MUPI in this case is very small. Depending on the sample the excess energy

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deposited in the molecule ions of the nucleobases was less than 0.7 eV up to less than 1.7 eV.

Besides the signals of the molecular ions of the nucleobases at *m/z* 111 for cytosine, *m/z* 126 for thymine, *m/z* 135 for adenine, and *m/z* 151 for guanine in all mass spectra signals for $[M + H]^+$ were found one mass unit higher than the molecular ions. Ionization was performed with MUPI in the gas phase, a method that generates molecular cation radicals $M^{\cdot+}$ and no adduct ions. A proton transfer to a nucleobase after the neutral molecules have reached the ion source is unlikely due to the absence of collisions between molecules in this region of the instrument. Therefore the hydrogen must have been attached to the neutral nucleobases before ionization. Ions formed during the desorption and in the supersonic beam expansion where multiple collisions take place are separated by the repeller. Only neutral molecules can pass the repeller into the ion source. Proton attached nucleobases formed in the jet would also be separated by the repeller. Therefore an attachment of a hydrogen radical to the nucleobases during the desorption must have taken place to form a neutral [M + H] radical, which could pass the repeller to be postionized with MUPI. The proportions of [nucleobase + H]/nucleobase signal intensities were found to be 0.5 for T, 1.1 for A, 1.3 for C, and 1.5 for G under our experimental conditions.

As discussed above molecular ions of the nucleobases were obtained by producing neutrals in the gas phase and postionizing them with little excess energy. As conditions found in supersonic beam expansions are known to favor cluster formation the intention was to study cluster formation of neutral nucleobases in the gas phase. "Soft" MUPI should provide a powerful tool to probe the proportions of neutral nucleobases and nucleobase pairs. To examine the dimer formation behavior we desorbed mixtures of two bases at a time. Mass spectra of all 10 possible nucleobase combinations were monitored. Mixtures of two bases at a time were preferred to examine instead of mixtures of three or four bases at a time to avoid any competition controlled formation of the nucleobase pairs. Typical mass spectra of at a time two nucleobases are shown in Figure 2, A and T (Figure 2 top) and C and G (Figure 2 bottom). Besides the signals for the molecular ions of the nucleobases the $[M + H]^+$ also show up in the mass spectra. As in both spectra homo- as well as heteronucleobase pairs can be seen, we have successfully monitored nucleobase pairs in the gas phase.

As our intention was to investigate the cluster behavior of neutral nucleobases, the region where the clusters are formed has to be addressed. There are no collisions between the molecules in the ion source and in the flight tube of the RETOF-MS due to the low density and directed translational movement of all particles. As no ionic material is able to pass the repeller only neutral species formed in the desorption chamber can enter the ion source. The nucleobase pairs are formed in the desorption chamber during the desorption of the nucleobases into a supersonic beam of argon. In the jet the translational velocity of all molecules is very similar due to multiple collisions between jet gas atoms and sample molecules. Therefore weak bonded dimers can be formed by the collision of two nucleobases. However, jet cooling provides the conditions to form and stabilize a nucleobase pair during the collision of two nucleobases. Without jet cooling a collision would lead to an unstable cluster which decays immediately due to the extent of internal energy. Due to multiple collisions of monomers nucleobase clusters are formed during the desorption process. This distribution of different clusters and monomers is "frozen" by the argon jet, and after a flight time of 300 μs the neutral nucleobases and nucleobase pairs have reached the ion source where ionization by MUPI takes place. Since the reactions occurring during a laser desorption process are still under evaluation, it should be noted, however, that this distribution of dimers does not necessarily correspond to a true gas phase equilibrium, such as

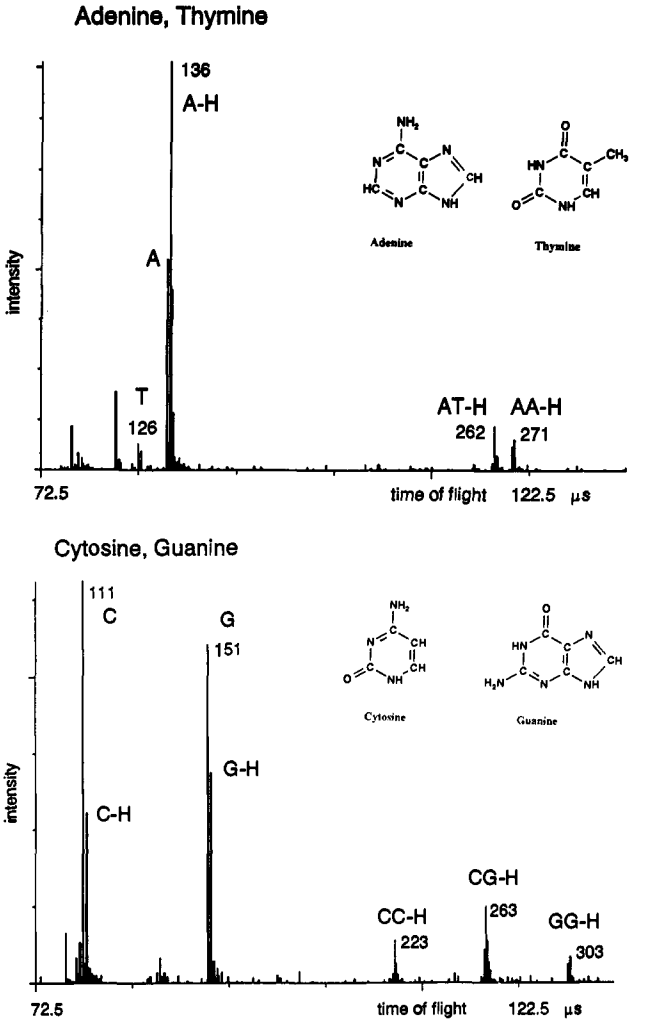

Figure 2. Time of flight mass spectra of mixtures of adenine/thymine (top) and cytosine/guanine (bottom). The spectra were obtained by post-ionization of neutral desorbed molecules with MUPI at 260 nm. Molecular ions, hydrogen attached ions, and cluster ions of the nucleobases can be seen.

$$B_1 + B_2 \rightleftarrows (B_1\text{–}B_2)$$

As the proportion of the neutral nucleobases and nucleobase pairs is of interest, the distribution of monomers and clusters in the jet should not be changed by the detection method. In our experimental setup TOF-MS is used as a detector. Therefore the distribution could be affected by the ionization process or cluster decay in the mass spectrometer. The excess energy deposited into the ions is less than 0.7 up to less than 1.7 eV as mentioned above. Furthermore the ions are detected after a time of flight of about 100 μs. Ionization conditions are required under which the ionized molecules and especially the ionized clusters remain stable for at least these 100 μs. Any decay of the clusters into the monomers or other fragments must be avoided in terms of relating the peak intensities of the detected ions to the proportion of the neutral nucleobases and nucleobase pairs. Fortunately nucleobase pairs are stabilized more in their ionic form than in their neutral form. By varying the wavelength of the ionization laser different amounts of excess energy could be deposited into the clusters. With ionization energies up to 10 eV, no significant dissociation of the nucleobase pairs could be observed within the time scale of our instrument. Furthermore, the intensities of both cluster and monomer peaks are independent of the laser intensity. To prove the absence of decomposing nucleobase pairs after ionization the metastable decay of the nucleobase pairs was observed by varying the electrostatic fields of the reflector. No

Table 1. "Pseudo Association Constants" for Nucleobase Pair Formation in the Gas Phase[a]

	K_a for free nucleobases	K_a for hydrogen attached
A-A	0.6	0.5
T-T	0.6	0.5
A-T	1.1	19
C-C	1.9	18
G-G	8.1	5
C-G	17	20
A-G	0.6	0.5
A-C	no A-C formation	no A-C-H formation
C-T	no T-C formation	no T-C-H formation
G-T	no G-T formation	no G-T-H formation

[a] Derived from experimental data under the assumption that collisions between jet cooled nucleobases may form nucleobase pairs. The unit of the K_a values is 10^{-6}/rel peak intensity

significant metastable decay of the nucleobase pairs to nucleobases or [nucleobase + H] could be seen. Therefore ionization at conditions described above provides a conservative method to detect the proportions of the neutral nucleobases and nucleobase pairs formed in the supersonic expansion.

In order to compare the stability of the different clusters the observed peak intensities of monomers and dimers were used to estimate so called "pseudo association constants" according to the following equation

$$K_a = [B_1{-}B_2]/\{[B_1][B_2]\}$$

The calculation of these "pseudo association constants" depends crucially on the assumption of a true gas phase equilibrium. However, it should be noted that, as the ionization efficiencies differ for different nucleobases, the calculated "pseudo association constants" are only estimations, not exact equilibrium constants.

Table 1 shows the "pseudo association constants" calculated from the experimental data. As can be seen the values for free nucleobases and hydrogen attached clusters vary by a factor up to 20. Due to the fact that the bonding in both cluster types might be different, it is not astonishing that the K_a values differ.

The values of the "pseudo association constants" displayed show that indeed an increased stability is observed for the nucleobase pairs. There are significant differences in the K_a values found for the formation of the nucleobase pairs. As not all nucleobase pairs are formed with the same K_a and a formation of A-C, C-T, and G-T heterodimers was not seen at all, base stacking is not likely to be a major contribution to the stabilizing forces of nucleobase pairs in the gas phase. Base stacking is not nucleobase specific. If base stacking or π-complexes of the nucleobases would play an important role in stabilizing the nucleobase pairs, a significant difference in the K_a values for the different nucleobase pairs should not be observed. Therefore other forces, such as hydrogen bonds must stabilize the nucleobase pairs in the gas phase.

Looking at the K_a values for the complementary nucleobase mixtures A/T and C/T a greater value is seen for the heterodimer formation than for the homo dimer formation. The formation of an A-T nucleobase pair is preferred to the formation of A-A or T-T homo dimers. The K_a value for the formation of A-T is twice as high as for the formation of A-A or T-T. Collisions between free nucleobases A and T in the gas phase result in a significantly higher amount of A-T nucleobase pairs than A-A or T-T nucleobase pairs. A specifity in nucleobase pair formation can be seen. Hence the double hydrogen bonded A-T nucleobase pair found in DNA is not only formed because of steric reasons due to the phosphate-sugar chain but the nucleobases A and T show a natural affinity themselves.

The other nucleobase pair found in DNA, C-G, is stabilized by three hydrogen bonds in the Watson–Crick configuration (Figure 1, 2). The gas phase K_a value found for C-G is the highest of all nucleobase pairs. The formation of C-G is preferred to the formation of the homodimers C-C and G-G. The nucleobase pair C-G is formed with the highest abundance of all nucleobase pairs. Nonempirical theoretical calculations have also determined the three hydrogen bonded C-G pair to be the most stable of all nucleobase dimers.[2] Our experimental results are in agreement with this theoretical calculation showing that heterodimers of complementary nucleobases are more stable than homo dimers. The opposite was found to be true for noncomplementary nucleobase pairs. For noncomplementary nucleobases homodimers are more stable than hetero dimers. The K_a value we found for C-C in the gas phase is greater than the K_a value for A-T, in good agreement to theory.[2,9]

These results can be interpreted in the formation of nucleobase pairs in the gas phase showing a significant specifity. However, as the errors of the K_a values are not known, the ranking of the "pseudo association constants" should not be overestimated. The trends of the K_a values we found in the gas phase can be compared with a partial list of values estimated from gas phase field mass spectrometry results[15] as well as from experiments with substituted nucleobases in a nonpolar solvent.[27] Our K_a values derived from the investigation of unsubstituted nucleobase pairs however provide new results for base pair formation. The order of K_a values we found is C-G > G-G > C-C > A-T > A-A, T-T, A-G > A-C, C-T, G-T. The interactions of free nucleobases in the gas phase result in a favored formation of complementary nucleobase pairs which are found in the double helix of DNA. A specific coupling to A-T and C-G pairs has been found between free bases in the gas phase.

The mass spectra of the four nucleosides adenosine (Ad), cytidine (Cd), guanosine (Gd), and deoxythymidine (Td) were obtained using the experimental setup described above. Figure 3 displays the mass spectra of mixtures of two nucleosides. Mass spectra of mixtures of Ad/Td and Cd/Gd are shown. Beside the peaks of the molecular ions of the nucleosides and the nucleoside pairs, the mass spectra show peaks one mass unit higher than the molecular ions. As discussed for the free nucleobases, the neutral nucleosides catch a hydrogen radical during the desorption step. The proportion of [nucleoside + H]/nucleoside was 0.3 for Td, 0.8 for Gd, 5 for Cd, and 9 for Ad. The hydrogen catching behavior has changed when comparing the nucleobases with the nucleosides.

Besides the molecular ions and the cluster ions, however, a significant amount of fragmentation appeared in the mass spectra. The origin of these fragment ions has to be addressed before deriving "pseudo association constants" from the experimental data. The ionization conditions like laser wavelength and laser intensity were adjusted to exclude fragmentation reactions due to the ionization process. By checking for metastable decay during the flight time no evidence for significant fragmentation due to the ionization could be found. Due to the sample preparation however a fragmentation of the labile nucleosides during the desorption process was most likely. As described above, a mixture of pure samples without any additives was used to avoid any influence to the cluster formation by molecules other than the nucleosides themselves. No matrix and/or comatrix such as polyethylene, sugar, metal powder, or phosphate known to support desorption of intact molecules was used. This lack of any matrix resulted in partial decomposition of the nucleosides during desorption that we accepted rather than cluster formation between nucleosides and matrix molecules. Decomposition of the nucleosides in this region of the experiment however does not affect the calculation of the K_a values. The "pseudo association constants" calculated from the peak intensities of the nucleosides and nucleoside clusters as described above are shown in Table 2. (The discussion of the "pseudo association constants" for nucleobase clusters is also valid here, i.e. they should be regarded rather as an estimation of cluster stability than a true equilibrium constant.)

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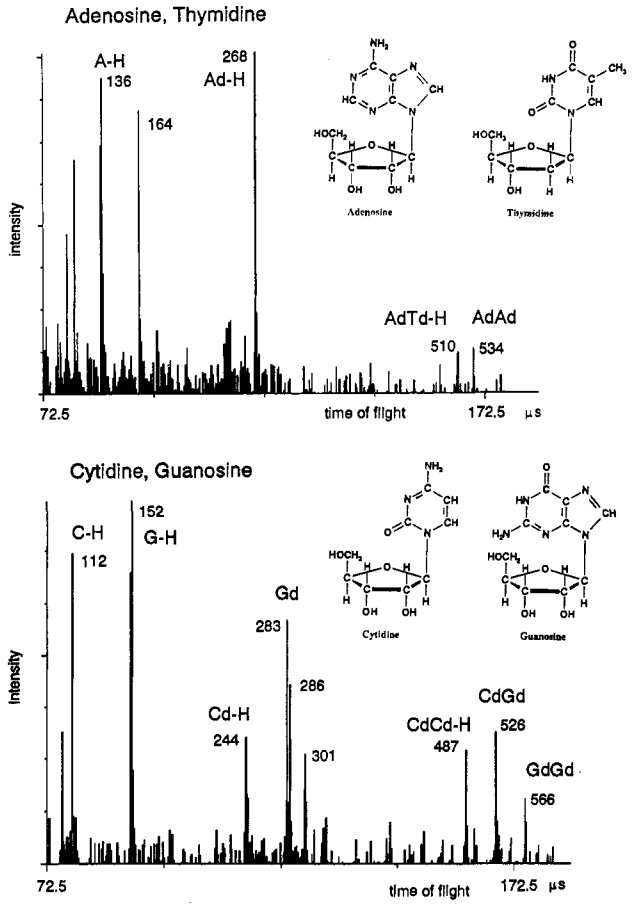

Figure 3. Time of flight mass spectra of mixtures of adenosine/thymidine (top) and cytosine/guanosine (bottom). Both spectra were obtained by post-ionization of neutral desorbed molecules with MUPI at 260 nm.

Table 2. "Pseudo Association Constants" for the Dimer Formation of Mononucleosides in the Gas Phase[a]

	K_a values for free mononucleosides	K_a values for hydrogen attached
Ad-Ad	200	0.5
Td-Td	0	0
Ad-Td	130	18
Cd-Cd	1300	400
Gd-Gd	870	50
Cd-Gd	2000	240

[a] K_a values are calculated from experimental data. The unit of the values is 10^{-6}/rel peak intensity.

The K_a values calculated for the nucleosides are generally higher than the "pseudo association constants" of the nucleobases. The preference of the dimer formation has changed. The complementary pair Cd-Gd is the most abundant of all dimers. The signals of the homodimers Cd-Cd and Gd-Gd are less intensive than the Cd-Gd heterodimer. By using the nucleosides, a preferred formation of Cd-Gd can be seen similar to the results for the nucleobases. In case of the nucleosides, the K_a value of Cd-Cd is greater than that of Gd-Gd, in opposition to the behavior of the free nucleobases. The homodimer Ad-Ad is formed with the highest abundance out of a mixture of Ad and Td. This result is in contrast to the nucleobase pairing which displays the anticipated behavior. Investigating the Ad/Td mixture, an even clearer change in dimer formation can be seen using nucleosides instead of nucleobases.

The changes made to the sample molecules were the addition of a ribose molecule to the nucleobases. Obviously the addition of a sugar molecule containing several hydroxyl groups influenced the cluster formation of the nucleosides in the gas phase. A strong modification of the affinities between the sample molecules could be found due to the side groups, an effect that has been often ignored in measurements of these affinities. As all possible hydrogen bonded configurations of A-T pairs are bound by two or one hydrogen bond, interactions are not as strong as in the C-G Watson–Crick pair. Therefore side chain effects are first observed at the A-T pair formation. A ribose molecule with some hydroxyl groups disturbed the native cluster behavior of A and T to alter the dominant formed dimer. Substituted nucleobases such as nucleosides may interact not only at positions of the nucleobases but also at positions of the side groups to form dimers thus reducing the nucleobase specifity to form complementary nucleobase pairs. The order of "pseudo association constants" was altered from the nucleobases as C-G > G-G > A-T > A-A to the nucleosides as C-G > C-C > G-G > A-A > A-T.

Conclusion

We have produced clusters of free nucleobases and mononucleosides in the gas phase. A supersonic beam expansion provided the conditions to form neutral clusters which were detected using MUPI as a conservative gentle ionization method. A natural affinity of unsubstituted nucleobases was found toward the formation of A-T and C-G heterodimers. The complementary nucleobase pairs A-T and C-G were formed favorably compared to all other possible hetero nucleobase pairs, a fact here shown for the first time in the gas phase. "Pseudo association constants" were derived from experimental results. The "pseudo association constant" for A-T was greater than for A-A or T-T as the "pseudo association constant" for C-G was greater than for C-C or G-G in the gas phase. The order of the "pseudo association constants" changed using mononucleosides instead of nucleobases. The side groups of the nucleosides provide additional linkage which may falsify the native affinities of the nucleobases. The order of the "pseudo association constants" of free nucleobases in the gas phase was determined to be C-G > C-C > G-G > A-T > A-A, T-T, A-G, > A-C, C-T, G-T. The results show that complementary nucleobase pairing in DNA is not only dependent on the phosphate–sugar chain but that the selectivity of the nucleobase pairs is already attributable to their native affinity.

We are aware that this are only preliminary results due to unsolved problems like different ionization efficiencies for different compounds, true gas phase equilibria, and development of cluster distributions with time.

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