Pair Formation of Free Nucleobases and Mononucleosides in the Gas Phase

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The formation of neutral bimolecular clusters of unsubstituted nucleobases and mononucleosides in the gas phase has been studied by IR laser desorption of the neutral molecules into a supersonic beam expansion. The complementary nucleobase pairs adenine-thymine and cytosine-guanine of DNA have been found to be formed in preference to non-complementary base pairs. 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 \( \pi \)-systems of the nucleobases of the two strands, steric forces due to the rigid backbones – the sugar-phosphate chains –, 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 was to study unmodified nucleobases and the corresponding mononucleosides in the gas phase in order to investigate whether the complementary nucleobase pairs have native affinities or are mediated by external effects. It turned out that it is important to study the nucleobase pair formation in absence of any side groups, which can be seen to lead to false conclusions.

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formed at other positions of the nucleobases. Co-crystal-
ization of adenine and thymine may result in for-
mation of A-T pairs in the Hoogsten geometry [10] (Figure 1.3). However, not only hydrogen bonds may
stabilize isolated nucleobase clusters, but also van der
Waals interactions. Overlapping of the \( \pi \)-systems of
nucleobases causes the formation of stacks of planar
parallel nucleobases (Figure 1.4). The van der Waals
interactions, however, have only a limited specificity, so
that a main formation of complementary base pairs
due to these forces could not be observed. Stacked
base pairs are 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 be-
tween the nucleobases but due to the influence of wa-
ter molecules [12, 13].

Several experimental investigations of substituted
nucleobase pairs in the gas phase [14] and in different
solvents [15, 16, 17, 18] have been performed. The
proportion of substituted nucleobases and nucleobase
dimers in solution was determined by IR spectroscopy
[19, 20] and NMR studies [21, 22]. In general, experi-
ments have shown that a hydrogen bonded configura-
tion of nucleobase pairs is more favored in non-polar
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.

To examine the contribution of the nucleobases
themselves to the forces that cause the formation of
definite complementary base pairs the interactions of
free nucleobases in the gas phase have been investi-
gated. We used nucleobases without substituting or
solvent to exclude solvent effects and side group
interactions. We felt that by investigating the free nu-
cleobases in the gas phase, the old question can be
solved, whether the nucleobases themselves form A-T
and C-G pairs or whether the formation of the Wat-
sen-Crick pairs is due to the three dimensional struc-
ture of the DNA or other external effects. The free
unsubstituted bases were chosen to exclude any side
group interactions, as they will be shown to influence
the nucleobase pair formation decisively.

Nucleosides, such as adenosine (Ad), deoxythy-
midine (Td), cytosine (C) and guanosine (G) differ
from the nucleobases by addition of a ribose molecule
to the N1 atom at the pyrimidine bases or to the N9
atom at the purine bases. As will be shown, the cluster
behavior of gaseous nucleosides differs decisively from
the cluster behavior of the nucleobases. This change
will be referred to side group effects affecting the dimer
formation of nucleosides.

Experimental

Experiments were performed with a reflection time
of flight mass spectrometer (RETOF-MS) described in
detail in [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 sam-
ple through a 8 cm ZnSe lens. A typical desorption
radiation 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 clus-
ters 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 pre-
vented 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 multi photon ionization (MUPI).

Pulsed radiation at variable wavelength was provided
by the frequency doubled (BBO-crystal) output of an
excimer laser (XeCl, LPX100 Lambda Physik Göttin-
gen) pumped dye laser (FL 3002 Lambda Physik Göttin-
gen). 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 \( 2 \times 10^8 \) W/cm\(^2\) by neutral
density filters. The positive ions produced by MUPI
postionization 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 desorp-
tion. The samples were used without further purifi-
cation 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 these samples could be achieved without significant fragmentation. Desorption and ionization were adjusted to soft conditions resulting in minimized fragmentation in both steps. Adjusting the intensity of the desorption laser results in minimizing fragmentation during the desorption. The intensity of the ionizing laser was adjusted such that the neutral sample molecules would absorb only two photons and not three or more. The wavelength of the ionizing laser was adjusted to significant ionization without fragmentation for all four nucleobases. 260 nm was found to be a wavelength at which all four nucleobases could be ionized by absorbing two photons. Therefore two photon postionization was performed with 260 nm (4.77 eV) and $10^7 \text{ W/cm}^2$. 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 was found to be 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 is 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 deposited in the molecule ions of the nucleobases is 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⁺° 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 joined 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 signals 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. Guanine has been found to be the best radical catching molecule among the four nucleobases.

As discussed above, molecular ions of the nucleobases were obtained by forming neutrals in the gas phase and postionizing them with little excess energy. As the conditions found in supersonic beam expansions are known to provide 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 ten possible nucleobase combinations were monitored. Mixtures of two bases at a time were examined instead of mixtures of three or four bases at a time in order to avoid any competition controlled formation of the nucleobase pairs. The time of flight spectra of two simultaneous nucleobases, these are A and T (Fig. 2, top) and C and G (Fig. 2, bottom), are shown in Figure 2. Beside the signals for the molecular ions of the nucleobases also [M + H]⁺ shows up in the mass spectra. As in both spectra homo as well as hetero nucleobase pairs can be seen, we have successfully monitored nucleobase pairs in the gas phase. Weakly bonded clusters as nucleobase pairs have been observed.

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 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 weakly bonded dimers can be formed by the collision of two nucleobases. There are multiple collisions in the region of the supersonic beam expansion. Collisions between two nucleobases, which often take place in this region, may result in the formation of nucleobase pairs. Only jet cooling, however, 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 its internal energy. Due to the multiple collisions the equilibrium of the nucleobase pair formation is built up faster than the desorption time of about 100 μs. The neutral nucleobases and nucleobase pairs are drifting then during about 300 μs into the ion source. There they are ion-ized by MUPI to cation radicals.

As the proportion of the neutral nucleobases and nucleobase pairs is of interest, the distribution should not be changed by the detection which includes ionization. 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 stay 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. To
Fig. 2. Time of flight mass spectra of mixtures of adenine/thymine and cytosine/guanine. The spectra were obtained by postionization of neutral cytosine/guanosine. Both spectra were obtained by postionization of neutral cytosine/guanosine.

Fig. 3. Time of flight mass spectra of mixtures of adenosine/thymidine and cytosine/guanosine. Both spectra were obtained by postionization of neutral cytosine/guanosine.
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 significant metastable decay of the nucleobase pairs to nucleobases 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.

From the peak intensities we calculated the association constants for the formation of the nucleobase pairs out of the free nucleobases and out of the hydrogen attached nucleobases in the gas phase. Due to the experimental parameters, the unit of the association constants given in this work is \(10^{-6}/\text{rel. peak intensity}\). The association constants for the nucleobase pair formation are calculated on the assumption that there is the following equilibrium:

\[
B_1 + B_2 = (B_1 - B_2),
K_a = \frac{[B_1 - B_2]}{[B_1][B_2]}
\]

with \(B_1\) and \(B_2\) representing free nucleobases in the gas phase forming the nucleobase pair \((B_1 - B_2)\) by collisions. \(K_a\) is the association constant derived from the intensities of the time of flight peaks \([B_1], [B_2]\) and \([B_1 - B_2]\) of the nucleobases \(B_1\) and \(B_2\) and the nucleobase pair \((B_1 - B_2)\). The association constant is derived assuming that collisions between jet cooled nucleobases may produce nucleobase pairs. The association constants are calculated by dividing the detected amount of the nucleobase pairs by the detected amount of the nucleobases. This provides a normalization of the values for the association constants. Any different ionization efficiencies of the four nucleobases at 260 nm would therefore not affect the values of the association constants.

Table 1 shows the association constants calculated from the experimental data. The values of the association constants displayed show that nucleobase pairing in the gas phase is nucleobase specific. 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 hetero dimers 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 \(\pi\)-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 significantly greater value is seen for the hetero dimer 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 specificity in nucleobase pair formation can be seen. Hence the double hydrogen bonded A-T nucleobase pair found in DNA is not only formed for 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 homo dimers C-C and G-G. The nucleobase pair C-G is formed with the highest abundance of all nucleobase pairs by collisions of neutral nucleobases in the gas phase. Theoretical calculations have also shown the threefold hydrogen bonded C-G pair to be the most stable of all nucleobase dimers [2]. Our results agree with the theoretical prediction that hetero dimers of complementary nucleobases are more

<table>
<thead>
<tr>
<th>Nucleobase Pair</th>
<th>(K_a) for free nucleobases</th>
<th>(K_a) for hydrogen attached</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-A</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>T-T</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>A-T</td>
<td>1.1</td>
<td>19</td>
</tr>
<tr>
<td>C-C</td>
<td>1.9</td>
<td>18</td>
</tr>
<tr>
<td>G-G</td>
<td>8.1</td>
<td>5</td>
</tr>
<tr>
<td>C-G</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>A-G</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>A-C</td>
<td>no A-C formation</td>
<td>no A-C-H formation</td>
</tr>
<tr>
<td>C-T</td>
<td>no T-C formation</td>
<td>no T-C-H formation</td>
</tr>
<tr>
<td>G-T</td>
<td>no G-T formation</td>
<td>no G-T-H formation</td>
</tr>
</tbody>
</table>
stable than homo dimers. The opposite was found to be true for non complementary nucleobase pairs. For non complementary nucleobases homo dimers are more stable than hetero dimers. The $K_a$ value we found for C-C in the gas phase is greater than $K_a$ for A-T, in good agreement with theoretical calculations [2, 9].

Our results show a significant specificity in the formation of nucleobase pairs in the gas phase. The trends of the $K_a$ values we found in the gas phase have to be compared to list values estimated from gas phase field mass spectrometry results [15] as well as from experiments with substituted nucleobases in a non polar solvent [27]. Our $K_a$ values derived from experiments in the gas phase with jet cooling and M UPI applied to 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 dominant formation of complementary nucleobase pairs which are found in the double helix of DNA. Molecules in the gas phase may perform rotations round all axes without steric hindrance. Therefore approach of the nucleobases in all geometric configurations is possible before the collisions. Nevertheless the formation of the complementary nucleobase pairs was found to be dominant. 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 at a time two nucleosides. Mass spectra of mixtures of Ad/Td and Cd/Gd are shown. Beside the peaks of the molecular ions of the nucleosides the mass spectra show peaks one mass unit higher than the molecular ions. As discussed with the free nucleobases, the neutral nucleosides catch a hydrogen radical in the gas phase. 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 comparing the nucleobases with the nucleosides. Within the nucleosides, Ad is the best radical catching molecule. In both mass spectra nucleoside pairs can be seen. We successfully monitored nucleoside clusters in the gas phase.

Beside 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 association constants from the experimental data. The ionization conditions as laser wavelength and laser intensity were adjusted to exclude fragmentation reactions due to the ionization process. The adjustment was proved by looking for fragment ions formed by 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 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. Therefore a matrix and/or comatrix such as polyethylen, sugar, metal powder or phosphate, known to support desorption of intact molecules, was not used. This resulted in partial decomposition of the nucleosides during desorption, which we rather accepted 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 association constants calculated as described above from the peak intensities of the nucleosides and nucleoside clusters are shown in Table 2. The $K_a$ values calculated for the nucleosides are generally higher than the association constants of the nucleobases. The preferences of dimer formation have changed. The complementary pair Cd-Gd is the most abundant of all dimers. The signals of the homo dimers Cd-Cd and Gd-Gd are less intensive than the Cd-Gd hetero dimer. Using the nucleosides, a preferred formation of Cd-Gd can be seen, the same as using the nucleobases. In case of the nucleosides, the $K_a$ value of Cd-Cd is greater then that of Gd-Gd.

| Table 2. Association constants for dimer formation of mononucleosides in the gas phase. $K_a$ values are calculated from experimental data. The unit of the values is $10^{-6}$/rel. peak intensity. |
|-----------------|-----------------|
|                 | $K_a$ values for free mononucleosides in the gas phase | $K_a$ values for hydrogen attached mononucleosides in the gas phase |
| 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 |
Con contrary to the behavior of the free nucleobases. The homo dimer 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 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 addition of a ribose molecule to the nucleobases, which increased the size of the sample molecules. Obviously the addition of a sugar molecule containing several hydroxy 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 one or two hydrogen bonds, interactions are not as strong as in the C-G Watson-Crick pair. Side chain effects are therefore first seen at the A-T pair formation. A ribose molecule with some hydroxy groups disturbed the native cluster behavior of A and T to alter the dominantly 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. The nucleobase specificity to form complementary nucleobase pairs is diminished by side groups, as the side groups themselves may cause interactions between two molecules. The order of association constants altered from the nucleobases as C-G > G-G > C-C > A-T > A-A to the nucleosides as C-G > C-C > G-G > A-A > A-T.

Conclusion

We produced clusters of free nucleobases and mononucleosides in the gas phase. A supersonic beam expansions provided the conditions to form neutral clusters which were detected using MUPI as a conservative ionization. A natural affinity of unsubstituted nucleobases was found towards the formation of A-T and C-G dimers. The complementary nucleobase pairs A-T and C-G were formed dominantly compared to all other possible hetero nucleobase pairs, a fact here shown for the first time in the gas phase. Association constants were derived from the experimental results. The association constant for A-T was greater than for A-A or T-T, as the association constant for C-G was greater than for C-C or G-G in the gas phase. The order of the association constants changed using mononucleosides instead of nucleobases. The side groups of the nucleosides provide additional linkage which may supersede the native affinities of the nucleobases. The order of the 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 the complementary nucleobase pairing in DNA is not only dependent on the phosphate-sugar-chain but also on the native affinity of the nucleobases.

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