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Effect of methylation on the pyrimidine–pyrimidine stacking interaction studied by ^{1}H NMR chemical shift

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Abstract

Mutually induced proton chemical shift changes were measured for the mixed solutions of pyrimidine and its methylated forms in deuterium oxide at 35°C. The chemical shift vs. concentration profiles were analyzed using a three-state decomposition model based on competitive self- and hetero-association dimer equilibria. The equilibrium constants show an increasing association tendency within the series pyrimidine–5-methyl-pyrimidine (0.23 ± 0.02 M^{-1}) < pyrimidine–4,6-dimethyl-pyrimidine (0.32 ± 0.04 M^{-1}) < 5-methyl-pyrimidine–4,6-dimethyl-pyrimidine (0.51 ± 0.04 M^{-1}). The upfield dimer shifts suggest an offset stacked geometry for the structure of associations between the parent molecule of the pyrimidine nucleobases and its methylated derivatives in aqueous solution.

Keywords: ^{1}H NMR chemical shift; Stacking interaction; Hetero-association; Methylation; Pyrimidine

1. Introduction

In addition to the four major bases present in DNA a small proportion of methylated bases may also be present. The commonest species of these minor bases are 6-N-methyladenine and 5-methylcytosine. Although the naturally added methyl group on specific bases does not appear to alter the basic rules of complementary base-pairing, they can bring about changes to the structural and dynamic properties of DNA [1,2]. The methylated adenine provokes e.g. hairpin [3] and bulged-out [4] structures and, similarly to thymine, to the methylated major base [5], the methylated cytosine [6] induces alteration in the magnitude of DNA curvature. The two methylated minor bases affect the thermal stability of duplexes [7]: while 6-N-methyladenine causes a decrease in melting temperature [8,9], the presence of 5-methylcytosine has the reverse effect [10,11], the increase in melting point is commensurable with that found for duplexes containing thymine in place of uracil [12]. These phenomena can be attributed to the changes in the base–base interactions, which can be decomposed into horizontal hydrogen bonding and vertical stacking interactions. The two types of interaction can be separated and modelled at the 'monomer base level' in the appropriate environments: hydrogen bonding is most pronounced in non-polar solvents where base stacking is negligible, and base stacking dominates in water where base–base hydrogen bonding is greatly suppressed [13,14]. The cru-

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cial difference between the two minor bases originates from the character of the methylated sites: adenine is methylated at the exocyclic amino group taking part in the cyclic hydrogen bonds in base-pairs, cytosine is methylated at an indifferent site concerning the base-pairing via hydrogen bonding. Consequently, it seems to be probable that the methylation of cytosine does not modify the base-pairing scheme, at least in the first approximation, and its effect on the duplex stability can be ascribed to the changes in the stacking interaction with nearest-neighbour bases. In order to elucidate the origin of the base–base stacking interaction, the self-association of purine, as one of the parent molecules of nucleobases, and its methylated derivatives in aqueous solution has been extensively studied by different experimental techniques [13–15]. While these experiments have demonstrated quantitatively that ring methylation increases the association tendency of purine, indirect evidence has been found for the self-association of the unmodified parent molecule of the pyrimidine nucleobases [16] and an experiment has indicated the hetero-association of pyrimidine and 5-methyl-pyrimidine in organic solution [17].

As the continuation of our previous work dealing with the effect of methylation on the self-association of pyrimidine [18], in this work, the cross-interactions between pyrimidine, 5-methyl-pyrimidine and 4,6-dimethyl-pyrimidine in mixed aqueous solutions were studied by measuring the mutually induced proton chemical shift changes. In order to characterize the non-, hemi- and bis-methylated associations of pyrimidine, the chemical shift (of the 'monitor' molecule) vs. concentration (of the 'inductor' molecule) profiles were analyzed using a three-state dimer model. The intention was to ascertain how the ring methylation affects the pyrimidine–pyrimidine stacking interaction.

2. Experimental and methods

Pure and mixed deuterium oxide solutions of pyrimidine (Sigma), 5-methyl-pyrimidine (Serva) and 4,6-dimethyl-pyrimidine (Aldrich) were prepared by dilution of stock solutions. (Chemicals were used without further purification.) Within a set of solutions the molar concentration of one solute (monitor) was kept constant at 0.1, 0.3 and 0.5 M while the other (inductor) was varied from 0.0 to 0.5 M; the samples with one solute served as external references.

The experimental procedure and the data analysis were described in detail previously [19]. The concentration-dependent chemical shift changes measured in solutions with two interacting solutes were interpreted as a result of molecular hetero-association between two self-associating compounds by means of dynamic NMR line-shape analysis in the fast exchange limit at equilibrium [20,21]. The partial line-shape analysis, i.e. the decomposition of the observed proton chemical shift (δ_{obs}) to the chemical shifts in monomer, self-associated and hetero-associated states (δ_m, δ_d and δ_c, respectively) was based on simultaneous, competitive dimer equilibria:

$$A + A \rightleftharpoons D1(=A_2)\ K_{d1} = [D1]/[A]^2$$

$$B + B \rightleftharpoons D2(=B_2)\ K_{d2} = [D2]/[B]^2$$

$$A + B \rightleftharpoons C(=AB)\ K_c = [C]/[A][B]$$

where K_{d1}, K_{d2} and K_c are the appropriate equilibrium constants. If $[A]_0 = [A] + 2[D1] + [C]$ and $[B]_0 = [B] + 2[D2] + [C]$ correspond to the total concentrations of the monitor and inductor molecules and if the monomer shift is used as reference, i.e. the experimental data are corrected for self-association occurring in reference solution, the chemical shift change vs. concentration profiles can be described by the equation:

$$\Delta_{corr} = 2\frac{[D1]}{[A]_0}\Delta_d + \frac{[C]}{[A]_0}\Delta_c \qquad (1)$$

where $\Delta_{corr} = \delta_m - \delta_{obs}$, $\Delta_d = \delta_m - \delta_d$ and $\Delta_c = \delta_m - \delta_c$; Δ_{corr} is the corrected upfield shift, Δ_d and Δ_c denote the self- and hetero-association dimer shifts, respectively (a negative sign corresponds to a downfield shift).

The ^{1}H NMR spectra were recorded at 60 MHz using a Perkin-Elmer R12A spectrometer at a probe temperature of 35°C. 'Best-fit' values in the least-squares sense for the equilibrium constants and for the dimer shifts were derived from Eq. (1) by data processing on a microcomputer system.

3. Results and discussion

As the self-association parameters for pyrimidine, 5-methyl-pyrimidine and 4,6-dimethyl-pyrimidine (henceforth Py, 5-MePy and 4,6-diMePy, respectively) in aqueous solution were determined previously [18], the mutually induced proton chemical shift changes measured in the Py–5-MePy, Py–4,6-diMePy and 5-MePy–4,6-diMePy aqueous systems can be standardized or corrected by referring the signals of the ring and methyl protons to the shifts at infinite dilution in pure solution. These extrapolated monomer shifts proved to be 548.1, 528.6 and 455.8 Hz for the Py protons (H-2, H-4,6 and H-5, respectively), 535.0, 519.0 and 140.4 Hz for the 5-MePy protons (H-2, H-4,6 and 5-Me, respectively) and 528.3, 148.3 and 439.8 Hz for the 4,6-diMePy protons (H-2, 4,6-Me and H-5, respectively) from an external 0.05 M deuterium oxide solution of DSS (at 60 MHz). In order to make the concentration dependency of the chemical shift changes more pronounced, the standardized or so-called corrected experimental data are shown in Figs. 1 and 2 instead of the direct experimental data; the data sets are translated along the ordinate in such a way that the intercepts correspond to the self-association upfield shifts of the references.

It can be seen from Fig. 1 that, on increasing the inductor concentration at a fixed monitor concentration, the corrected upfield shifts of the protons in the counterparts increase selectively, and from Fig. 2 that, at different monitor concentrations, the data sets are convergent and saturable. Furthermore, it is remarkable that the saturation rate increases on going from the Py–5-MePy system to the Py–4,6-diMePy and 5-MePy–4,6-diMePy systems. Assigning the concentration-dependent changes in chemical shifts to the changes in equilibrium population of the non-associated and associated states, the model leading to Eq. (1) seems to be suitable for deriving the hetero-association parameters from the chemical shift vs. concentration profiles. The equilibrium constants and the dimer shifts with their standard deviations for the hetero-associations (and for the self-associations from Ref. [18]) are presented in Table 1.

Since the values of the individual hetero-association constants, calculated for each of the protons of the counterparts, and the values of the average het-

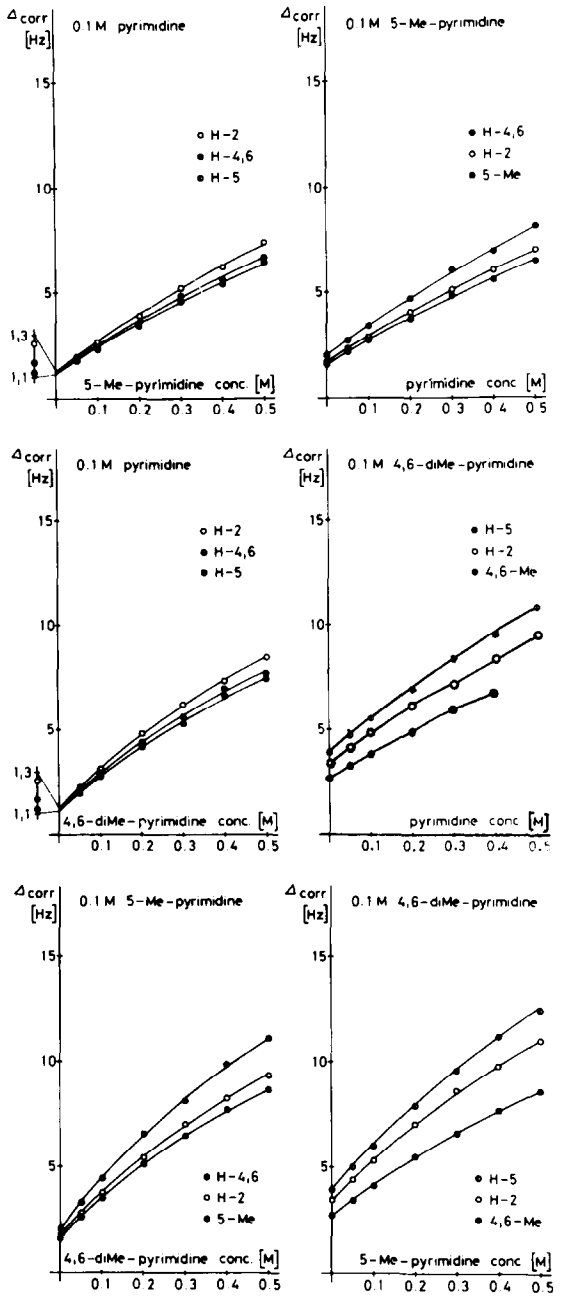

Fig. 1. Chemical shift vs. concentration profiles for the ring and methyl protons in pyrimidine–5-methyl-pyrimidine, pyrimidine–4,6-dimethyl-pyrimidine and 5-methyl-pyrimidine–4,6-dimethyl-pyrimidine mixed deuterium oxide solutions at a fixed concentration of the 'monitor' molecule. Corrected experimental (symbols) and fitted (solid lines) upfield shifts relative to the extrapolated monomer shifts in pure solutions; temperature, 35°C.

ero-association constants, calculated separately for the counterparts, are the same within the standard deviation, one can determine the so-called overall average equilibrium constants, which were found to be 0.23 ± 0.02, 0.32 ± 0.04 and 0.51 ± 0.04 M^{-1} for the Py–5-MePy, Py–4,6-diMePy and 5-MePy–4,6-diMePy associations, respectively. The trend of the equilibrium constants indicate that methylation significantly enhances the association ability of pyrimidine in water. Comparison of the self- and hetero-association constants implies that the association tendency depends on the degree of methylation and on the 'counterparts' asymmetry' existing in the hetero-associations.

The hetero-association dimer shifts support the earlier results obtained for the self-associations and clarifies some further features of the effect of methylation on the pyrimidine–pyrimidine stacking interaction. From their positive values, it can be stated that the associated pyrimidines are in each other's diamagnetic shielding regions, hence they have a plane-to-plane or stack-like arrangement [22,23] with an association axis perpendicular to the parallel planes of the interacting molecules. The striking feature of the data is that in various associations there is no significant change in the relative values of the dimer shifts of the same monitor molecule. Therefore, starting from the assumption that, in a shielding region having a slightly distorted 'conic symmetry', the dimer shifts for the protons at different positions in the same molecule reflect the horizontal distance from the association axis and their changes in various associations can be attributed to the vertical separation of the associated molecules, it is possible to outline probable or time-averaged association structures.

A number of characteristic properties can be drawn from the individual dimer shifts. First, in the dimer states H-2 is the most shielded ring proton of Py and the less shielded ring proton of 5-MePy and 4,6-diMePy. Second, the methyl protons of 5-MePy 'feel' a fairly large diamagnetic shielding effect. Third, none of the three pyrimidines having C_{2v} symmetry shows any sign of a change in the magnetic equivalence at the 4- and 6-positions, where the methyl protons of 4,6-diMePy are much less shielded than the ring protons of Py and 5-MePy. These findings can be explained by the assumption that the associa-

Table 1
Equilibrium constants in M^{-1} and upfield dimer shifts in Hz (at 60 MHz) for self-association (K_d and Δ_d) [18] and hetero-association (K_c and Δ_c) in pure and mixed solutions of pyrimidine, 5-methyl-pirimidine and 4,6-dimethyl-pyrimidine, calculated from the concentration-dependent self-induced and mutually induced chemical shift changes using dimer models (solvent, deuterium oxide; temperature, 35°C)

Protons of monitor molecules	Inductor molecules					
	Pyrimidine		5-Methyl-pyrimidine		4,6-Dimethyl-pyrimidine	
	K_d	Δ_d	K_c	Δ_c	K_c	Δ_c
Pyrimidine	0.091 ± 0.006 [a]		0.22 ± 0.01 [a]		0.33 ± 0.03 [a]	
H-2	0.096 ± 0.011	72.0 ± 2.8	0.21 ± 0.01	70.3 ± 2.8	0.34 ± 0.05	70.5 ± 1.5
H-4,6	0.085 ± 0.007	66.7 ± 2.5	0.21 ± 0.02	64.3 ± 2.6	0.35 ± 0.02	64.5 ± 2.6
H-5	0.092 ± 0.010	63.9 ± 2.2	0.23 ± 0.01	61.2 ± 2.0	0.32 ± 0.02	61.8 ± 3.1
	K_c	Δ_c	K_d	Δ_d	K_c	Δ_c
5-Methyl-pyrimidine	0.24 ± 0.02 [a]		0.165 ± 0.006 [a]		0.51 ± 0.03 [a]	
H-2	0.24 ± 0.01	58.1 ± 1.7	0.168 ± 0.008	54.6 ± 1.1	0.51 ± 0.03	52.1 ± 1.0
H-4,6	0.25 ± 0.02	67.6 ± 2.6	0.170 ± 0.010	64.9 ± 1.7	0.51 ± 0.03	61.8 ± 1.3
5-Me	0.22 ± 0.02	54.2 ± 2.7	0.157 ± 0.010	51.4 ± 1.9	0.52 ± 0.04	48.2 ± 2.1
	K_c	Δ_c	K_c	Δ_c	K_d	Δ_d
4,6-Dimethyl-pyrimidine	0.30 ± 0.04 [a]		0.50 ± 0.04 [a]		0.410 ± 0.011 [a]	
H-2	0.32 ± 0.05	56.7 ± 2.0	0.52 ± 0.03	51.0 ± 0.9	0.425 ± 0.022	48.4 ± 2.1
4,6-Me	0.29 ± 0.04	46.2 ± 2.1	0.50 ± 0.06	40.1 ± 1.4	0.414 ± 0.015	37.7 ± 1.1
H-5	0.30 ± 0.02	64.9 ± 1.4	0.49 ± 0.02	58.1 ± 1.0	0.392 ± 0.021	55.4 ± 1.9

[a] Average equilibrium constants.

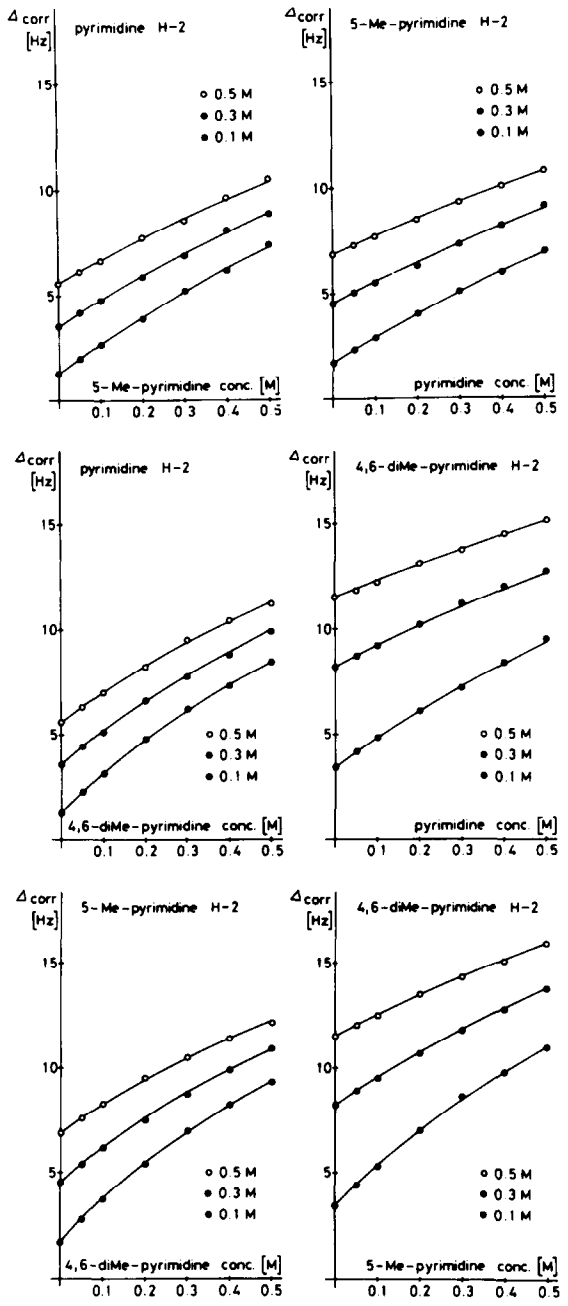

Fig. 2. Chemical shift vs. concentration profiles for the H-2 protons of pyrimidine, 5-methyl-pyrimidine and 4,6-dimethyl-pyrimidine in mixed deuterium oxide solutions at different concentrations of the 'monitor' molecule. Corrected experimental (symbols) and fitted (solid lines) upfield shifts relative to the extrapolated monomer shifts in pure solutions; temperature, 35°C.

tion axis shifts along the symmetry axis, C-2–C-5, from the nitrogen site towards the methylated site of the two methylated molecules. The relationships of the dimer shifts can be understood if the symmetry axes of two associated molecules are anti-parallel in the only non-methylated association (Py–Py self-association) and in the three bis-methylated associations (5-MePy–5-MePy and 4,6-diMePy–4,6-diMePy self-associations and 5-MePy–4,6-diMePy hetero-association), but they are parallel in the two hemi-methylated associations (Py–5-MePy and Py–4,6-diMePy hetero-associations), and if shifted (or partial) overlap takes place at the nitrogen site of Py and at the methylated site of 5-MePy and 4,6-diMePy. According to this interpretation, the association axis passes through the inversion centre describing the self-association structures, and this centre is over/under the nitrogen site of the unmethylated pyrimidine and over/under the methylated site of the methylated pyrimidines. Although such a definite symmetry element cannot be attributed to the hetero-association structures, it is expected that methylation increases the overlap area both for self- and hetero-associations.

The dimer shifts given in the rows of Table 1 show that the unmethylated pyrimidine induces the largest shielding effect which becomes smaller when the inductor is methylated pyrimidine. The decreasing tendency is the most pronounced for the dimer shifts of dimethylated pyrimidine. Comparison of the dimer shifts within the columns of Table 1 seems to be rather artificial. However, it is clear that the dimethylated pyrimidine induces the largest changes for the protons of identical position in the three molecules. The trend of the changes in dimer shifts can be accounted for by two effects: the higher electron density accompanying methylation leads to a 'shielding-increasing' ring current effect and the bulky methyl group, owing to its steric demand, results in a 'shielding-decreasing' steric effect. Thus, the dimer shifts can be interpreted as the manifestation of the two effects of opposite sign. In the non-methylated association, the Py–Py dimer shifts imply the vertically most packed association structure. In the hemi-methylated associations, corresponding to the monitor–inductor relationship, the Py–5-MePy and Py–4,6-diMePy dimer shifts suggest that the ring current effect almost compensates

the steric effect, whereas the 5-MePy–Py and 4,6-di-MePy–Py dimer shifts show the dominant role of the unmethylated pyrimidine. In the bis-methylated associations, the relationships of the dimer shifts 5-MePy–5-MePy > 5-MePy–4,6-diMePy and 4,6-di-MePy–5-MePy > 4,6-diMePy–4,6-diMePy, refer to the fact that the steric effect overcomes the ring current effect. These results indicate that the vertical separation of the molecules associated via partial overlap correlates with the degree of methylation.

Without question, the total association energy can be partitioned into contributions arising from different types of interaction. The magnitude of the association constants and their dependency on the degree of methylation suggest that hydrophobic interaction is one of the main driving forces of pyrimidine associations in water. The dispersion interaction can also make a considerable contribution to the magnitude of the stacking interaction between heterocyclic aromatic molecules. However, since both of them favour total overlap [24], they cannot be the interaction which results in partial overlap. Consequently, because induction interaction is generally a second-order term, the supposed association arrangement can be attributed to electrostatic interaction. Indeed, so-called offset stacked geometry has been established by a model [25] based on the 'distributed multipole analysis' method [26]. The model, separating the σ-framework and the π-electrons of the interacting (either polarized or non-polarized) aromatic molecules, demonstrates that the net $\pi-\pi$ interaction is actually the result of $\pi-\sigma$ attraction that overcomes $\pi-\pi$ repulsion and predicts an offset or slipped geometry for the association structure.

4. Conclusions

According to the 'NMR time-window', the decompositional model applied here describes some of the features of the effect of methylation on the pyrimidine–pyrimidine interaction in water through average dimerization substitution of the probably various actual association modes. However, the adequacy of this model, at least for comparison, is supported by the nearly equal hetero-association constants obtained separately for the counterparts. The equilibrium constants and the 'shielding map data' have shown that methylation increases the association proclivity of pyrimidine and modifies the shifted plane-to-plane association structure. On the basis of the results obtained at the 'monomer base level', it may be assumed that the methyl group is not a passive 'spacer' that alters the molecular geometry in nucleic acids, but instead works as an active element that causes, through changes in the strength of base–base stacking interaction, a higher duplex stability.

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