DOES 3′-CYTIDINE MONOPHOSPHATE BOUND TO RIBONUCLEASE A ACQUIRE *SYN*-CONFORMATION?

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1. Introduction

The accepted mechanism of action of RNAase A, consistent with the X-ray diffraction studies of RNAase and its nucleotide complexes, suggests an *anti*-conformation of the nucleotide in the substrate–enzyme complex [1]. The recent PMR investigations of 3′-CMP–RNAase A complex in solution were interpreted to indicate that the nucleotide is in *anti*-conformation at pH < 6, but it has to be in *syn*-conformation at pH > 6. Since the maximum reaction-rate is found at pH ~ 7, the authors consider such a change in the conformation as functionally significant [2]. Assuming base orientation to be directly related to the conformation of the ribose-ring, one can presume that *syn*-orientation of the base implies a planar-conformation of the ribose-ring. The strain-energy thus induced by RNAase A in the substrate molecule could be utilized to increase the rate of the reaction [2]. However, this determination of base-orientation about the glycoside-bond seems ambiguous, since the conclusions were based exclusively on the analysis of the $H_{1'}$ chemical-shifts of the 3′- and 5′-CMP bound to the enzyme.

The nuclear Overhauser effect measurements have proved to be an effective tool for evaluating the conformation of free 3′-CMP in solution and when bound to RNAase [3,4]. Our present work employs NOE in order to compare directly the nucleotide conformation of the 3′-CMP–RNAase A complex at p*D*-values of 5.0 and 6.7. If *syn–anti* isomerization of 3′-CMP in the complex were the case, the conformation of 3′-CMP bound to the enzyme should be significantly different at these p*D* values, since p$K \sim 6$ was assigned to the isomerization [2].

Abbreviations: NOE nuclear Overhauser effect, RNAase A bovine pancreatic ribonuclease, PMR proton magnetic resonance

2. Materials and methods

Chromatographically pure samples of RNAase A and 3′-CMP (sodium salt) were used. The exchangeable protons of RNAase A were replaced with deuterium. To this end, the protein sample was dissolved in D_2O (concentration 0.2%) and was kept at p*D* 3.5 and a temperature of 30°C for two days, then the solution was lyophilized.

NMR Samples were prepared with 0.02 M RNAase solutions and 0.016 M or 0.012 M solutions of 3′-CMP at p*D* either 5.0 or 6.7. The substances were dissolved in 0.2 M NaCl in D_2O (p*D* 5.5). The 'p*D*' (uncorrected pH-meter reading) was adjusted with 1 M NaOD or DCl to 5.0 or 6.7.

The free nucleotide concentration in the complex solutions did not exceed 3%, as the binding constant for 3′-CMP to RNAase A equals 7.5×10^3 M and 5.0×10^3 M at pH 5.0 and 6.7, respectively [5]. The NMR spectra were obtained at 100 MHz using a Varian spectrometer HA-100D at 30°C. The experimental technique for the NOE measurements has been described previously [3,6]. The computation of the NOE-values $f_{H_{1'}}(H_6)$ as a function of the torsional glycosidic-angle was performed as was described in our earlier publication, taking into account that correlation-time for the dipole–dipole interaction of nucleotide protons (τ_c) was equal to 3.0×10^{-8} s [4]. To calculate τ_c, we used known experimental data on

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the nucleotide resonance line-width for 3′-CMP bound to RNAase [7], and the original equation for the estimation of relaxation time (T_2) was employed [8].

3. Results and discussion

The determination of the nucleotide conformation in the 3′-CMP–RNAase A complex, defined by the torsional glycosidic angle φ_{CN}, was carried out by measuring the NOE between H_6 and $H_{1'}$ protons, which depends strongly on φ_{CN}. The NOE enhancements were recorded for the $H_{1'}$ protons upon saturation of the H_6 proton-line of the nucleotide (fig.1), and were found to be equal to -0.06 ± 0.02 (pD 5.0) and to -0.04 ± 0.02 (pD 6.7). The analysis of the NOE data was based on the theoretical correlation between the NOE-values $f_{H_{1'}}(H_6)$ and the angle φ_{CN} (fig.2). The calculations were performed assuming that both the base and the ribose-ring of the 3′-CMP are rigidly bound to the protein.

The distances between $H_{1'}$ and H_6 protons at different φ_{CN} values were obtained using data on the structure of the 3′-CMP in the crystalline state when

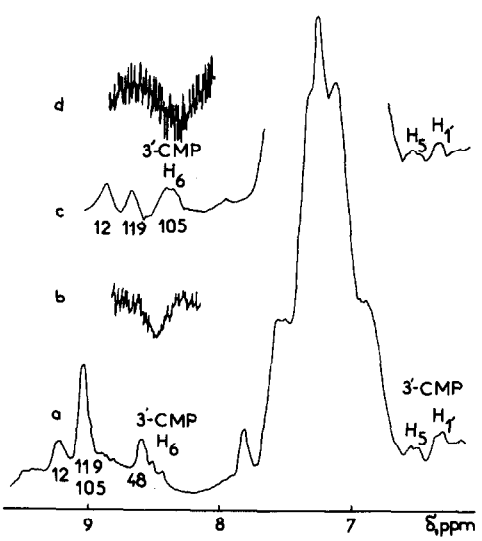

Fig.1. ^{1}H-NMR- and NOE-spectra of 3′-CMP–RNAase A complex. Aromatic absorption-region of the complex at pD 5.0 (a) and pD 6.7 (c). NOE for $H_{1'}$ upon saturation of H_6 at pD 5.0 (b) and pD 6.7 (d).

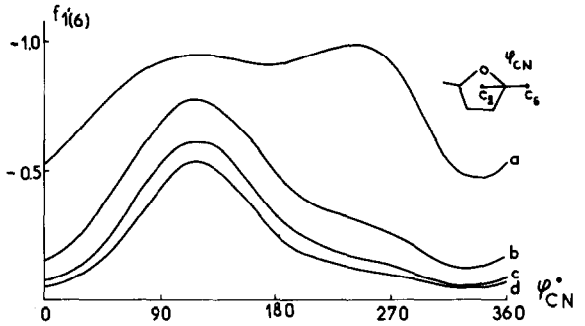

Fig.2. The NOE enhancements for $H_{1'}$ of 3′-CMP bound to RNAase A upon saturation of H_6 computed as a function of glycosidic torsion angle. No interaction between $H_{1'}$ and any of protein-protons is assumed (a). $H_{1'}$ interacts with one proton (b), two protons (c), and three protons (d) of the enzyme, which are situated at a distance of 2.4 Å. The correlation-time for the dipole–dipole interaction of nucleotide-protons (τ_c) of 3′-CMP in the complex is equal to 3.0×10^{-8} s [4].

the ribose ring was in 3′-endo-conformation [9]. NOE-Values calculated for 3′-CMP having a planar ribose-ring, geometry of which in the crystalline state was given in [10], were similar to those for 3′-endo ribose. Consequently, the selected conformation of the ribose-ring did not affect the accuracy of the computation.

It is clear from the data obtained that the NOE enhancement-values $f_{H_{1'}}(H_6)$ for the 3′-CMP–RNAase A complex at pD 5.0 and 6.7 are similar. The theoretical correlation enables one to find that the corresponding angle φ_{CN} is equal to $330 \pm 25°$, and two or three protein-protons are located quite near the $H_{1'}$ nucleotide-proton. The calculated angle $\varphi_{CN} = 330°$ (or either $\varphi_{CN} = -30°$) shows that 3′-CMP in the complex is in the *anti*-conformation at both pD values. The similarity of the NOE-values at pD 5.0 and pD 6.7 can be taken as an indication that the corresponding φ_{CN}-values lie within the 50°-range (stipulated by accuracy of measurements). In the case of *syn*-conformation, one should expect to find $f_{H_{1'}}(H_6) \leqslant -0.34$.

Gorenstein and Wyrwicz concluded, having studied the pH-dependency of H_6-, H_5- and $H_{1'}$-resonances of the 3′-CMP and 5′-CMP bound to RNAase A, that the 3′-CMP in the complex underwent the pH-dependent transition from *anti*- to *syn*-conformation

[2]. They assumed a priori that the $H_{1'}$ chemical-shift depends mostly on the base carbonyl-group orientation in relation to $H_{1'}$ (keto-group anisotropy-effect) and the protein influence on $H_{1'}$-resonance of a nucleotide in each of the complexes studied is either identical or does not exist at all. As a matter of fact, however, according to the X-ray diffraction study of the enzyme–nucleotide complexes [1] and the data obtained for the complexes in solution [4], the ribose-ring is indeed involved in the direct interaction with the protein in the enzyme–nucleotide complex. Moreover, we have shown that, at p*D* 5.5, the C2-H-proton of His-12 in the complex is positioned at about 3.6 Å from the $H_{1'}$-proton of 3′-CMP, i.e., the imidazole ring of His-12 is adjacent to the protons in question [4]. At p*D* ~ 6, the His-12-residue of the complex is charged positively. Therefore, slight variations in orientation of the imidazole-ring of His-12 in relation to the ribose should cause alterations in the $H_{1'}$-proton chemical-shift – due to the change in the distance to the charged-group and to the change of the anisotropic influence of its ring-current.

Thus, our results demonstrate that in the 3′-CMP–RNAase A complex, the nucleotide base has an *anti*-orientation in respect to the ribose-ring within the p*D*-range 5.0–6.7. Consequently, the 'conformational' RNAase A mode of action suggested by Gorenstein and Wyrwicz [2] cannot be reconciled with the experimental data on the 3′-CMP–enzyme complex structure.

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