Determination of the nucleotide conformation in the productive enzyme-substrate complexes of RNA-depolymerases

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Abstract The aim of this work is to determine the conformation of the nucleobase adjacent to the cleavable phosphodiester bond in the productive enzyme-substrate complex of RNA-depolymerizing enzymes. To this end the kinetic parameters of hydrolysis of UpA, 2′-C-Me- and 3′-C-Me-UpA were determined for RNase A, RNase Pb_2, nuclease S_1 and snake venom phosphodiesterase. In these derivatives the ranges of the allowed orientation of uridine residues are restricted due to the substitution of methyl groups for the ribose hydrogen atoms. The results described demonstrate that the proposed method is of general value for the estimation of the nucleotide glycoside angles in the productive enzyme-substrate complexes.

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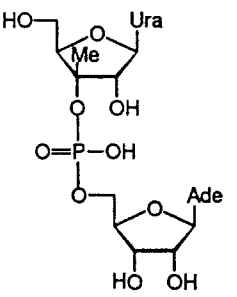
Key words: RNA-depolymerizing enzyme; C′-methyl analogues of UpA; Conformation of substrate

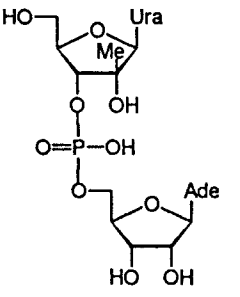
1. Introduction

Study of the molecular mechanisms of enzyme action includes the design of a model of the productive enzyme-substrate complex and a description of the subsequent events during product formation. The modeling of a productive enzyme-substrate complex is presently based mainly on the results of X-ray and NMR investigations of the enzyme complexes with inhibitors. In the case of RNA cleaving enzymes, monoribonucleotides [1–5], short oligodeoxynucleotides [6,7] or dinucleoside phosphates whose stability was achieved by replacement of reacting groups [1,8,9] are mainly used as the substrate analogs. The question arises as to the relationship between the structures of the enzyme-inhibitor complex and the enzyme-substrate complex. For instance, in studies of the complexes of RNase *St. aureofaciens* with 3′-GMP, 2′-GMP and guanosine 2′,3′-cyclophosphorothioate by the X-ray method it was observed that the nucleobases in the complexes are fixed in the *anti* conformation with respect to the ribose ring [10–12]; however, it was suggested that in the productive enzyme complex with guanosine 2′,3′-cyclophosphate the nucleobase is bound in the *syn* conformation. Similarly, for RNase *B. amyloliquefaciens* the observed X-ray structures of the enzyme complexes with d(GpC) [13] and d(CGAC) [7] are different from the suggested structure of the true enzyme-substrate complex [2,7]. The same is true for the structure of the RNase A complex with d(CpG) [14].

The determination of the nucleotide conformation in the productive enzyme-substrate complex is the important step in development of the molecular model of action of RNA cleaving enzymes. With this aim we performed a comparative study of the enzyme hydrolysis rate of natural substrates and their derivatives having the restricted glycosidic conformations due to substitution of methyl groups for ribose protons. Here the use of 2′-C-Me- and 3′-C-Me-UpA for the estimation of the uridine conformation of UpA in the enzyme-substrate complexes is considered using four RNA depolymerizing enzymes: RNase *Penicillium brevicompactum* (RNase Pb_2), RNase A, nuclease S_1 and snake venom phosphodiesterase (PDE).



3′-C-Me-UpA



2′-C-Me-UpA

2. Materials and methods

Bovine pancreatic RNase A (type XII-A) was a Sigma product. UpA, nuclease S_1 and snake venom phosphodiesterase were purchased from Serva. RNase Pb_2 was prepared as described [15]. The concentrations of enzymes and substrates were determined spectrophotometrically using the following extinction coefficients: $\varepsilon_{280} = 10\,400$ M^{-1} cm^{-1} for bovine pancreatic RNase [16], $\varepsilon_{280} = 44\,300$ M^{-1} cm^{-1} for RNase Pb_2 [17], $\varepsilon_{261} = 23\,500$ M^{-1} cm^{-1} for UpA and its derivatives [17]. The concentrations of nuclease S_1 and snake venom phosphodiesterase were indicated by Serva on the flask. RNase activity was measured by spectrophotometric assays performed in 0.1 M sodium citrate, pH 6.2, containing 0.1 M NaCl at 25°C. Absorbance changes associated with substrate cleavage were continuously monitored and converted to initial reaction velocities by using the difference in molar absorption coefficients $\Delta\varepsilon_{286} = 570$ M^{-1} cm^{-1} [17] for RNase A and RNase Pb_2. The difference molar coefficient of UpA and its derivatives for nuclease S_1 and snake venom phosphodiesterase was determined by the complete hydrolysis studies and was equal to 2900 M^{-1} cm^{-1} at 265 nm. Spectral and kinetic determinations were made with a thermostatically controlled Specord-M40 Spectrophotometer (Carl Zeiss, Germany). The width of the optical slit was adjusted to 0.6–1.2 nm. Cells with an optical path

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length of 0.2 cm and 1 cm were used. Kinetic parameters were determined by the Lineweaver-Burk plot. The date were analyzed using a weighted least-squares procedure.

3′-*C*-Methyluridilyl-(3′-5′)-adenosine (3′-C-Me-UpA) was prepared by condensation of 5′-*O*-benzoyl-3′-*C*-methyluridine [18] and N^6-acetyl-2′,3′-di-*O*-acetyladenosine 5′-phosphate in the presence of *N*,*N*′-dicyclohexylcarbodiimide followed by removal of acyl blocking groups and separation of 3′-5′ and 2′-5′ isomers on DEAE-cellulose using a concentration gradient of NH_4HCO_3 (0.0–0.1 M). Analogously starting from 5′-*O*-benzoyl-2′-*C*-methyluridine [19] 2′-*C*-methyluridilyl-(3′-5′)-adenosine (2′-C-Me-UpA) was prepared. The purity of the synthesized dinucleoside phosphates was checked by HPLC.

All calculations were performed at fixed bond lengths and angles in accordance with Arnott and Hukins [20]. We used algorithms described earlier for calculating the amplitude of pseudorotation corresponding to the optimal form of a furanose cycle at variable P angle [21]. The methyl group was considered a tetrahedron with standard C-H bonds lengths and free rotation around the C-C bond. Conformational energetic maps (the influence of pseudorotation angle *P* and glycoside angle χ on potential energy kcal/mol) were calculated for uridine and its 2′-*C*- and 3′-*C*-methyl derivatives. The maps were created for free rotation around exocyclic bond C4–C5 and were almost identical to the maps for uridine 3′-phosphate and its C′-methyl derivatives. In calculations we used various sets of force parameters. For all cases we obtained very similar energetically forbidden areas $E_{analog} - E_{naturalnucleoside} > 5$ kcal/mol caused by mutual interaction of the methyl group and the uracil base.

3. Results and discussion

The interaction of the substrate and enzyme in solution may result in the formation of a number of structurally different complexes, but only the definite type of complex is productive. The introduction of the methyl groups in the 2′- or 3′-positions of the ribose moiety of nucleotide significantly narrows the regions of possible base orientations.

The methyl group substitution for protons in the ribose residue results in nucleoside analogs, having all functional groups of natural compounds, e.g. all possible binding sites for the enzymes of nucleic acid biosynthesis. As a result, comparable binding constants for analogs and natural compounds can be expected. Two extreme cases of the applicability of this approach may be considered: (1) the analog is transformed and is well bound with the enzyme, so for the conformation of the substrate in the enzyme-substrate complex the condition ($E_{analog} \approx E_{naturalnucleoside}$ is satisfied); (2) the analog is not transformed by the enzyme, then the conformation of the substrate in productive enzyme-substrate complex must be sought in the region where $E_{analog} \gg E_{naturalnucleoside}$, provided that the introduction of the voluminous methyl group causes intramolecular, and not intermolecular steric hindrance [22].

To detect intramolecular contacts between the methyl group and the heterocyclic base, conformational analysis of nucleotides and their analogs using the force-field method with 'rigid' bond length approximation was carried out (Fig. 1). The energy barrier of the *syn-anti* conversion is noticeably higher when the methyl group is introduced in the 2′-position (Fig. 1B). Only *N*-conformers of 3′-C-Me-Urd have energetically forbidden conformations, whereas no intramolecular collisions occur in *S*-conformers (Fig. 1C). Taking as allowed conformations for the uridine (Fig. 1A) moiety of the substrate in the enzyme-substrate complex those for which the calculated energy value of nucleoside does not exceed that for the global minimum by more than 5 kcal/mol, one can draw the following conclusions. For pseudorotation phase angles of a sugar ring $P = 0$–$36°$, corresponding to the family of *N*-conformers, the allowed values of glycoside torsion angle χ for the natural nucleoside in the enzyme-substrate complex belong to the regions 35–55° and 170–280°. For the family of the ribose *S*-conformers ($P = 144$–$180°$) these regions are 30–70°, 170–265° and 305–325°. For 3′-*C*-methyluridine derivatives intramolecular contacts were found only for the *N*-conformer family (Fig. 1C) and the energetically allowed χ values were 170–210° (Fig. 2). In the case of 2′-*C*-methyluridine the allowed χ region for *N*-conformers is 195–255° and for *S*-conformers 170–230°. This enabled us to find a comparatively small region of the allowed χ values and in some cases to determine the characteristic ribose type conformation based on the measurement of the enzyme reaction with the three types of substrates including uridine and its two *C*′-methyl derivatives.

The introduction of a methyl group into the C2′- or C3′-position of the nucleotide ribose moiety may change the reactivity of the respective internucleotide phosphodiester bond and may possibly change the ionization constant of the 2′-OH group in the transesterification reaction. Studies of the rates of nonenzymatic hydrolysis of 3′-C-Me-U(3′,5′)pA and 3′-C-Me-U(2′,5′)pA were carried out and compared to those of U(3′,5′)pA and U(2′,5′)pA (detailed results of alkaline hydrolysis of these dimers will be published elsewhere). The observed first-order rate constant of hydrolysis of 3′-C-Me-U(2′,5′)pA was almost equal to those of U(2′,5′)pA and

Table 1
Kinetic parameters of the hydrolysis reactions of UpA, 2′-C-Me-UpA, 3′-C-Me-UpA by RNase A, RNase Pb_2, nuclease S_1 and snake venom phosphodiesterase

Enzyme	Substrate	k_{cat} (s^{-1})	K_M (K_I) (μM)	k_{cat}/K_M (s^{-1} $M^{-1} \times 10^{-3}$)
RNase Pb_2	UpA	900	150	6000
	3′-C-Me-UpA	140	120	1200
	2′-C-Me-UpA	*	(780)	*
RNase A	UpA	1100	1100	1000
	3′-C-Me-UpA	510	2900	180
	2′-C-Me-UpA	*	(1300)	*
Reaction →		NpN′ → N > p+N′		
Nuclease S_1	UpA	180	590	300
	3′-C-Me-UpA	1.3	220	5.9
	2′-C-Me-UpA	9.7	1700	5.7
PDE	UpA	5.4	15	360
	3′-C-Me-UpA	2.1	57	37
	2′-C-Me-UpA	4.6	77	60
Reaction →		NpN′ → N+pN′		

*Enzymatic cleavage was not detected.

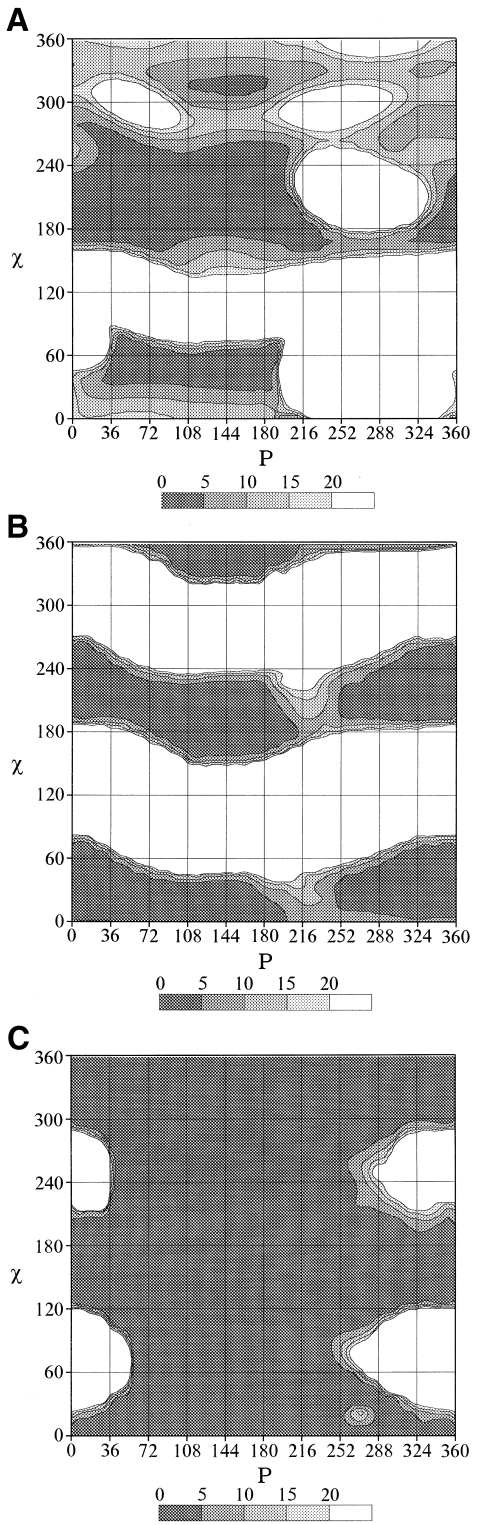

Fig. 1. Conformation energy maps in the χ-P space, isoenergy contours are drawn at 5 kcal/mol intervals. The first contour corresponds to 5 kcal/mol relative to the global minimum. A: Potential energy (E) for natural Urd; B: $E_{2'-C-Me-Urd}-E_{Urd}$; C: $E_{3'-C-Me-Urd}-E_{urd}$.

U(3′,5′)pA. The observed first-order rate constant of hydrolysis of 3′-C-Me-U(3′,5′)pA was about one order of magnitude greater than that of U(3′,5′)pA. These data enable us to conclude that the introduction of a methyl group into the C2′- or C3′-position does not decrease the reactivity of the corresponding internucleotide phosphate group in the trans-etherification reaction. So any substantial decrease of the corresponding enzyme reaction rate for C′-methyl derivatives of UpA may be attributed to steric contacts. This is in agreement with the fact that bimolecular constants k_{cat}/K_M for PDE for UpA derivatives decrease by not more than 10 times the same constants for UpA. It is known that this enzyme is not specific for the radicals flanking the phosphate.

The kinetic parameters of the hydrolysis reactions of UpA, 2′-C-Me-UpA and 3′-C-Me-UpA by RNase Pb_2, RNase A, nuclease S_1 and PDE are given in Table 1. For RNase Pb_2 the molecular rate constant k_{cat} of the cleavage reaction of 3′-C-Me-UpA is approximately 6 times lower than that for the native dinucleoside phosphate UpA with an almost unchanged K_M. Cleavage of 2′-C-Me-UpA was not observed, its inhibition constant is only 5 times greater than the K_M for the hydrolysis of UpA. Its stability towards the RNase Pb_2 action is most likely due to the nonproductive orientation of the uracil ribose phosphate fragment with respect to the enzyme catalytic residues in the enzyme-substrate complex arising from the inability to form a productive glycoside torsion angle χ. Based on the calculations shown in Fig. 1, these results allow us to draw the following conclusion about the value of χ in the productive enzyme-substrate complex. A minor change in the hydrolysis rate for 3′-C-Me-UpA suggests that χ may fall in the region of 170–210° for *N*-conformation and in the regions of 30–70°, 170–265° and 305–325° for *S*-conformers. The lack of cleavage for 2′-C-Me-UpA excludes the allowed values for 2′-C-Me-Urd. Hence, the productive values of χ may correspond to the regions of 30–70°, 230–265° and 305–325° for *S*- and 170–195° for *N*-conformers (the dotted gray field in Fig. 2).

For RNase A the molecular rate constant k_{cat} of the cleavage reaction of 3′-Me-UpA is only two times lower than that for the native dinucleoside phosphate UpA with an approximately equal increase of K_M. Cleavage of 2′-C-Me-UpA was not observed. The inhibition constant of the RNase A cleavage reaction of UpA by this derivative was almost equal to the K_M value of the hydrolysis reaction of UpA. This means that 2′-C-Me-UpA binds at the enzyme active site with a

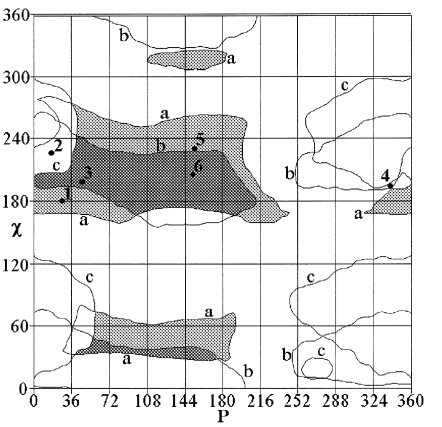

Fig. 2. Isoenergy contours corresponding to 5 kcal/mol relative to global minimum: a, E_{Urd}; b, $E_{2'-C-Me-Urd}-E_{Urd}$; c, $E_{3'-C-Me-Urd}-E_{urd}$. The conformations of pyrimidine nucleotides in the B_1 site of RNase A in crystal: 1, UpcA [3]; 2, 2′-CMP [4]; 3, uridine 2′,3′-vanadate [5]; 4, 3′-CMP [1]; 5, d(ATAA) [6]; 6, dCpA [1].

dissociation constant equal to the corresponding value for UpA and suggests nonproductive binding conformation as in the case of RNase Pb_2.

The conformations of pyrimidine nucleotides in the complexes with RNase A fit quite well to the area of allowed conformations of Urd and are shown in Fig. 2. In all of these crystal structures the uracil base is bound at the B_1 site [3] of the enzyme. Examination of these complexes reveals that the protein main chain (Phe-120 residue) is located close to the 2′- and 3′-protons of the pyrimidine nucleotide. In some cases the formal replacement of the corresponding protons by a methyl group results in the intermolecular steric contacts. So analysis similar to the foregoing may be restricted in this case only by conformations of 3′-C-Me-UpA.

In the case of nuclease S_1 the molecular rate constants k_{cat} of the cleavage reactions of 3′-C-Me-UpA and 2′-C-Me-UpA decrease 140 and 19 times, respectively, with relatively small changes in K_M. These results correspond to the single region of torsion angle χ values 190–210° for *N*-conformation and 170–230° and 30–40° for *S*-conformation of the ribose moiety (the dotted black field in Fig. 2).

The results described here demonstrate that the proposed method for the estimation of the nucleotide glycoside angles in the productive enzyme-substrate complexes of RNA-depolymerizing enzymes is effective.

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