STEREOCHEMISTRY OF THE TRANSESTERIFICATION STEP OF PANCREATIC RIBONUCLEASE

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Summary: Determination by X-ray analysis of the absolute configuration of uridine 3'-O-thiophosphate methyl ester obtained by the reaction of uridine 2',3'-O,O-cyclothiophosphate with pancreatic ribonuclease in aqueous methanol, establishes an in-line mechanism for the transesterification reaction of this enzyme.

Pancreatic ribonuclease A hydrolizes ribonucleic acids in a two-step mechanism, the first a transesterification step to a cyclic phosphate and the second a hydrolysis step opening the ring to a pyrimidine nucleoside 3'-phosphate. A number of mechanisms for these two reactions have been proposed (1), which basically can be classified as in-line or adjacent (2), as illustrated for the hydrolysis of uridine 2',3'-cyclophosphate in Fig. 1. Both mechanisms can be visualized as proceeding through a pentacoordinated phosphorous intermediate in the form of a trigonal bipyramid as discussed by Westheimer (3). The adjacent mechanism requires pseudo-rotation of this pentacoordinate phosphorous intermediate whereas the in-line mechanism does not. To distinguish between the two mechanisms by identification of the reaction product, we recently synthesized uridine 2',3'-O,O-cyclophosphorothioate (4), a compound with an asymmetric

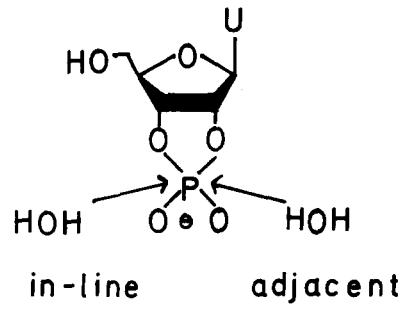

Fig. 1 Mechanisms for hydrolysis of uridine 2',3'-cyclophosphate.

phosphorous atoms of which both diastereomers are substrates for pancreatic ribonuclease (5). One of the two isomers could be crystallized as the triethylammonium salt and its absolute configuration was established by X-ray analysis (6). It is the endo-isomer which has the same K_m-value as uridine 2',3'-cyclophosphate and therefore is a suitable substrate analogue for an anylsis of the stereochemistry of the mechanism of action of RNase.

In this publication we demonstrate that the endo-isomer of uridine 2',3'-cyclothiophosphate can be used for an investigation of the mechanism of the first reaction step of RNase A.

EXPERIMENTALS AND RESULTS

3'-O-thiophosphate methylester

Incubation of the crystalline endo-isomer of uridine 2',3'-cyclothiophosphate (4) (282 mg, 5600 OD_{260} units, mp 203-205°) in 1.65 ml 0.1 M ethylene diamine buffer (pH 7.0) and 13 ml of methanol with crystalline RNase A (Boehringer + Söhne, Germany) (1.65 ml of a solution containing 2 mg of enzyme in 1 ml H_2O) for 4 hrs. at room temperature led to a mixture of unreacted starting material (R_F 0.59, 12 %), uridine 3'-thiophosphate (R_F 0.36, 30 %) and uridine 3'-thiophosphate O-methylester

(R_F 0.67, 57 %) as shown by paper chromatography (Schleicher & Schüll, 2043 b washed in isopropanol/2 N ammonia, 3 : 7 v/v). Separation by preparative paper chromatography yielded 3200 OD_{260} units of the methylester. By passage over a Merck ion exchange column (tri-ethyl ammonium

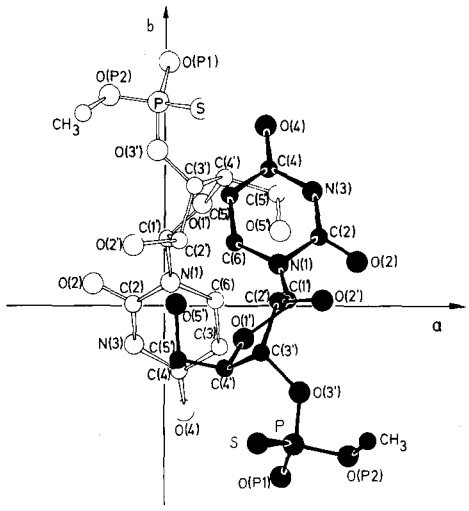

Fig. 2 Projection along the c-axis of one asymmetric unit of the crystal structure of uridine-3'-O-thiophosphate methylester; the locations of the disordered triethylammonium cations are not indicated. The two nucleotide molecules are of similar conformation due to a pseudo twofold screw axis parallel to the c-axis. For clarity the molecule in front has been shadowed.

form), evaporation of the eluate and dissolving the residue in hot isopropanol we obtained the methyl ester in crystalline form, yield 15 mg (2850 OD_{260} units, 51 %, mp 152-154° (tri--ethyl-ammonium salt)). The structure of this methylester was elucidated by X-ray analysis and is shown in fig. 2 which is equivalent to structure A in figure 3.

Uridine 2',3'-cyclothiophosphate from uridine 3'-O-thiophosphate methylester

Incubation of the crystalline methylester (30 mg, 650 OD_{260}

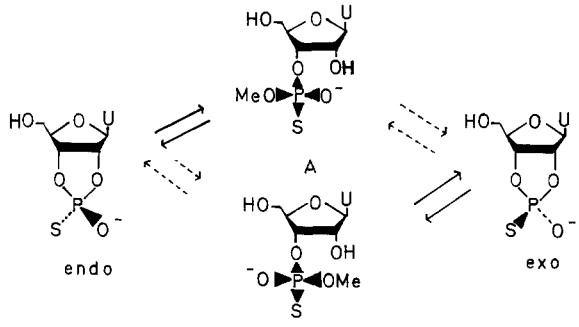

Fig. 3 Stereochemistry of transesterification of uridine-2',3'-cyclothiophosphate for in-line (⟶) and adjacent (- - - -→) mechanism.

units) with RNase (100 µl of a solution containing 2 mg enzyme in 2 ml H_2O) in 0.1 M triethylammonium bicarbonate buffer pH 7.5 (4 ml) for 2 hrs. at room temeprature led to a mixture of products [unreacted methylester (81 %), uridine 3'-thiophosphate (3 %) and uridine 2',3'-cyclothiophosphate (16 %)] as evidenced by paperchromatography. Purification of the uridine 2',3'-cyclothiophosphate combined from 2 such experiments by preparative paperchromatography and transformation into the triethylammonium salt yielded 125 OD_{260} units of material of which 112 OD_{260} units crystallized from isopropanol (mp 203-206°). This material is the endo-isomer (fig. 3) as shown by its melting point and by its X-ray diffraction pattern which is indistinguishable from that of the starting cyclothiophosphate endo-isomer (6).

Determination of molecular structure of uridine 3'-O-thiophosphate methylester

Long prismatic crystals were obtained by slow evaporation of an isopropanol solution of the triethylammonium salt of uridine 3'-O-thiophosphate methylester. The crystals all had very poor diffraction patterns. The space group is monoclinic, $P2_1$ and the cell dimensions as measured on a four circle diffractometer equipped with a Mo-tube (λ = 0.70926 Å) are

$a = 6.854_2$ Å, $b = 14.804_4$ Å, $c = 22.542_7$ Å, $\beta = 92.8_3$ °. From the cell dimensions and density the asymmetric unit contains two formula units (2 times $C_{19}H_{29}O_8N_3PS$). Because of the rapid decline of the X-ray intensities with increasing glancing angle only 2243 data up to $\theta = 20^{\circ}$ were collected. The data were converted to normalized structure factors, and the phase problem was solved by cyclic application of the tangent formula in a multi solution technique approach (7). The phase information of eight starting reflections (three for origin fixation, two from Σ_1-relationships, three additional reflections whose phases were allowed to vary by 45° and 180° respectively) was extended to include all the E's greater than 1.4. From the resulting 64 phase sets the most reliable was selected by consistency criteria. An E-map based on this phase set revealed the atomic positions of the two nucleotide molecules but not the triethylammonium cations. At the present stage the atoms of the cations can not be located unambiguously since they are apparently disordered. The residual index $R = \Sigma||Fo| - |Fc|| / \Sigma|Fo|$ is 32 % . Although the refinement of the structural details is far from complete, the essential features are clear.

The two molecules are related to each other by a pseudo-twofold screw axis which gives rise to almost identical conformational characteristics (figure 2).

The nucleotides both are in the anti-conformation with torsional angles C(2')-C(1')-N(1)-C(6) (8) about 70°. The sugar residues are in a C(2')-endo envelope form; atoms O(5') are located "above" the ribose moieties with both torsional angles C(3')-C(4')-C(5')-O(5') and O(1')-C(4')-C(5')-O(5') in the gauche form (about 40° and -70° respectively).

The conformation of the phosphodiester group for the two molecules is described by the torsional angle C(2')-C(3')-

O(3')-P, 160°, and C(4')-C(3')-O(3')-P, -85°. The angle C(3')-O(3')-P-S is about -40° and the methyl group is trans to atom O(P 1) but gauche to O(3') and the sulfur atom in both molecules.

The stereochemistry of the phosphodiester group is such that, when looking down the O(3') ⟶ P bond, S, O(P1) and O(P2)-CH_3 follow at 120° in a clockwise direction.

DISCUSSION

Findlay et al. (9) have shown that incubation of a pyrimidine nucleoside cyclophosphate in an aqueous alcohol solution with RNase A leads to appreciable transesterification and that the alcohol is competing with water in this reaction. With the endo-isomer of uridine 2',3'-cyclothiophosphate in aqueous methanol one obtains in this reaction uridine 3'-O-thiophosphate methylester with the absolute configuration as shown in Fig. 2. This is the isomer one expects from an in-line attack of methanol on the cyclothiophosphate (Fig. 3). The reverse reaction which proceeds in aqueous solution yields the endo-isomer of the cyclothiophosphate which also is entirely consistent with the in-line mechanism. It has been shown earlier with the endo-isomer (10) that the second step of RNase A action, the hydrolysis step also follows the in-line mechanism.

Inspection of the three dimensional model of the complex of the methylene analog of UpA with the enzyme (1) reveals that histidine 12 is next to the 2'-oxygen atom of uridine. An in-line mechanism requires 2 histidines, one in the acidic and one in the basic form. In the light of the information of the crystalline enzyme-inhibitor complex and the results described here, it is most reasonable to assume (Fig. 4) that in the ring opening reaction either by transesterification or hydrolysis (10) histidine 119 abstracts a proton from the incoming nucleophile-

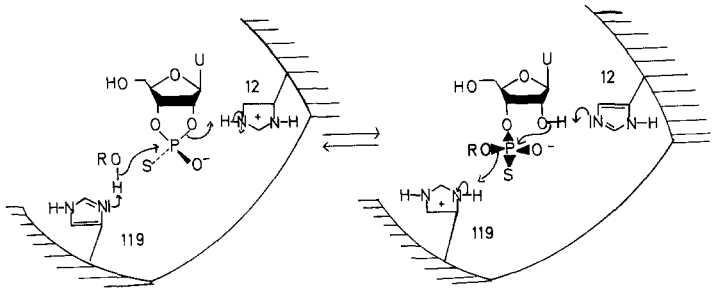

Fig. 4 Mechanism of transesterification by Pancreatic ribonuclease.

HOH or ROH- and histidine 12 protonates the 2'-oxygen. In the transesterification step to the cyclophosphate histidine 12 abstracts a proton from the 2'-oxygen and histidine 119 protonates the leaving group. This mechanism was first proposed by Findlay et al (11).

The approach described here for pancreatic ribonuclease is applicable to all nucleases which have a cyclophosphate as an intermediate and which accept cyclothiophosphates as substrates.

An account on the stereochemstry of the first step using cytidine instead of methanol is forthcoming (12).

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