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Geometry of the First Step in the Action of Ribonuclease-A

(in-line geometry/uridine 2′,3′-cyclic thiophosphate/^{31}P NMR)

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ABSTRACT **Advantage was taken of the reversibility of the first step of ribonuclease-A action to synthesize the dinucleoside phosphorothioate Up(S)C from the crystalline isomer of uridine 2′,3′-cyclic phosphorothioate [Uĝ(S)] and cytidine. Cyclic phosphorothioate was then reformed from Up(S)C by a nonenzymic reaction known to proceed by an in-line mechanism. The geometry of the enzymic reaction was determined to be in-line by a comparison of the Uĝ(S) product with the Uĝ(S) originally used. By the principle of microscopic reversibility, the geometry of the first step in the action of ribonuclease-A is shown to be in-line.**

Bovine pancreatic ribonuclease-A catalyzes the hydrolysis of a ribonucleic acid or nucleotide ester by a two-step reaction. A transesterification in which the 2′—OH group attacks one of the phosphodiester bonds to form a 2′,3′-cyclic phosphate is followed by the hydrolysis of this intermediate to yield a terminal 3′-phosphate (1). Each of these steps could proceed by either an in-line or an adjacent mechanism, depending on the relative geometry of the attacking and leaving groups in the displacement reaction (2). An adjacent mechanism requires the formation of a pentacoordinate intermediate, which must undergo pseudorotation before the product can be released.

Since Rabin's original proposal in 1961 (3), several mechanisms have been suggested (4–8). Most of these can be classified as in-line or adjacent by our combining a knowledge of the mechanism of hydrolysis of phosphate esters with an examination of the groups claimed to participate in the catalysis. For example, in the first step, we would infer an adjacent mechanism if the same group acts first as a general base to deprotonate the 2′—OH and then as a general acid toward the leaving ester group (2). Nuclear magnetic resonance (NMR) and x-ray evidence have been presented to support an in-line mechanism (9), but this conclusion, being based on the mode of binding of dianionic inhibitors, is equivocal. Until now, the most pertinent evidence favoring an in-line mechanism for the first step has been x-ray diffraction studies on a complex of the enzyme with a phosphonate analog of the natural substrate uridylyl-adenosine (10).

A conclusive determination of the geometry of the second step, in which the monoanionic diester uridine 2′,3′-cyclic phosphorothioate was used as substrate, has been reported (11); the mechanism was in-line. Although the ring-opening step is often shown as the microscopic reverse of the ring closure, the two steps need not have the same mechanism, since water is obviously not equivalent to a nucleoside. We have, therefore, extended our work to provide a conclusive determination of the geometry of the first step of this reaction.

Abbreviations: Uĝ, uridine 2′,3′-cyclic phosphate; Uĝ(S); uridine 2′,3′-cyclic thiophosphate; UMP(S), uridine 2′(3′)monothiophosphate; Up(S)C, uridylylthio 3′,5′-phosphorylcytidine; TLE, thin-layer electrophoresis.

EXPERIMENTAL

Materials. Bovine pancreatic ribonuclease-A was obtained as a lyophilized, phosphate-free powder from Worthington Biochemical Corp. Cytidine was from Sigma Chemical Co. Uridine 2′,3′-cyclic thiophosphate was prepared from 5′-*O*-acetyl uridine and trisimidazole phosphine sulfide by the method of Eckstein (12), as modified by Richardson (13), and purified as its triethylammonium salt by column chromatography. The crystalline diastereomer was separated from the mixture of crystalline and liquid isomers (Fig. 1) by recrystallization four times from 95% ethanol (mp 214–216°C with decomposition). The identity of the isomer was established qualitatively by optical rotatory dispersion (ORD), and quantitatively by ^{31}P NMR; the NMR technique indicated a maximum of 5% contamination by liquid isomer.

Methods. Purity of the starting material and the composition of the reaction mixture were determined by thin-layer electrophoresis (TLE) with 0.4 M triethylammonium bicarbonate buffer (pH 7.2), and by thin-layer chromatography (TLC) in 95% EtOH–1 M NH_4OAc 7:3 (v/v) (pH 7). All column chromatography was on DEAE-Sephadex A-25 resin (Pharmacia); a concave concentraion gradient of triethylammonium bicarbonate buffer (pH 7.2) was used for elution. Fractions were analyzed by measurement of their absorbance on a Cary 15 spectrophotometer, and pooled accordingly. Fourier-transform ^{31}P NMR was performed on a Bruker

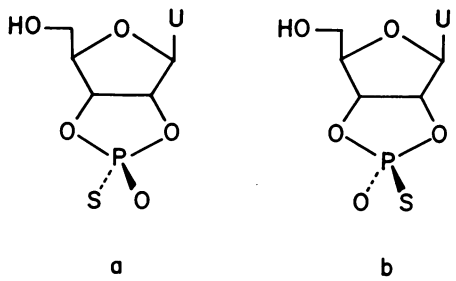

FIG. 1. The two diastereoisomers of uridine 2′,3′-cyclic phosphorothioate.

$$C + U\hat{p}(S) \overset{\text{RNase-A}}{\rightleftharpoons} Up(S)C$$

$$Up(S)C \xrightarrow[\text{In-Line}]{\text{Base}} U\hat{p}(S) + C$$

FIG. 2. The overall method used to test the geometry of the first step of ribonuclease action.

HX-90 machine, and ORD was on a Cary 60 spectropolarimeter.

Experimental Procedure. The enzyme-catalyzed reaction was performed at −18°C with 179 mg of Up̂(S), 2.0 g of cytidine, and 175 μl of a 0.45 mM solution of RNase, in a total volume of 15.2 ml of Tris acetate buffer (pH 6). TLE and TLC indicated that after 22 days the rate of formation of dinucleoside phosphate relative to the rate of hydrolysis of Up̂(S) began to level off, and the fraction of Up(S)C relative to UMP(S) began to decrease. The reaction was quenched with an excess of bentonite (5 times the weight of RNase) (14). Up(S)C was isolated by column chromatography and lyophilized. Its identity was established by comparison of its TLE and TLC behavior with that of a reference sample of UpC, and by RNase hydrolysis (1 mM substrate and 5 μM enzyme), which yielded Up̂(S) and C. Up(S)C was recyclized at room temperature in a nitrogen-filled glove bag, with an excess of 0.5 M potassium *t*-butoxide in *t*-butanol–formamide 43:57 (v/v). The reaction was quenched with acetic acid after 24 hr, and the resulting Up̂(S) was isolated by column chromatography. Its isomeric composition was determined by ^{31}P NMR and by ORD. The identity of the ^{31}P NMR peaks (of product) was checked by sequential addition of liquid isomer to the sample tube, the spectrum being rerun after each addition.

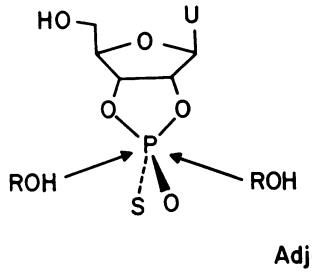

FIG. 3. The directions of attack for the in-line and adjacent mechanisms. ROH = Cytidine.

RESULTS

Overall method

The method used to test the geometry of the first step is shown in Fig. 2. The reversibility of the first step of ribonu-

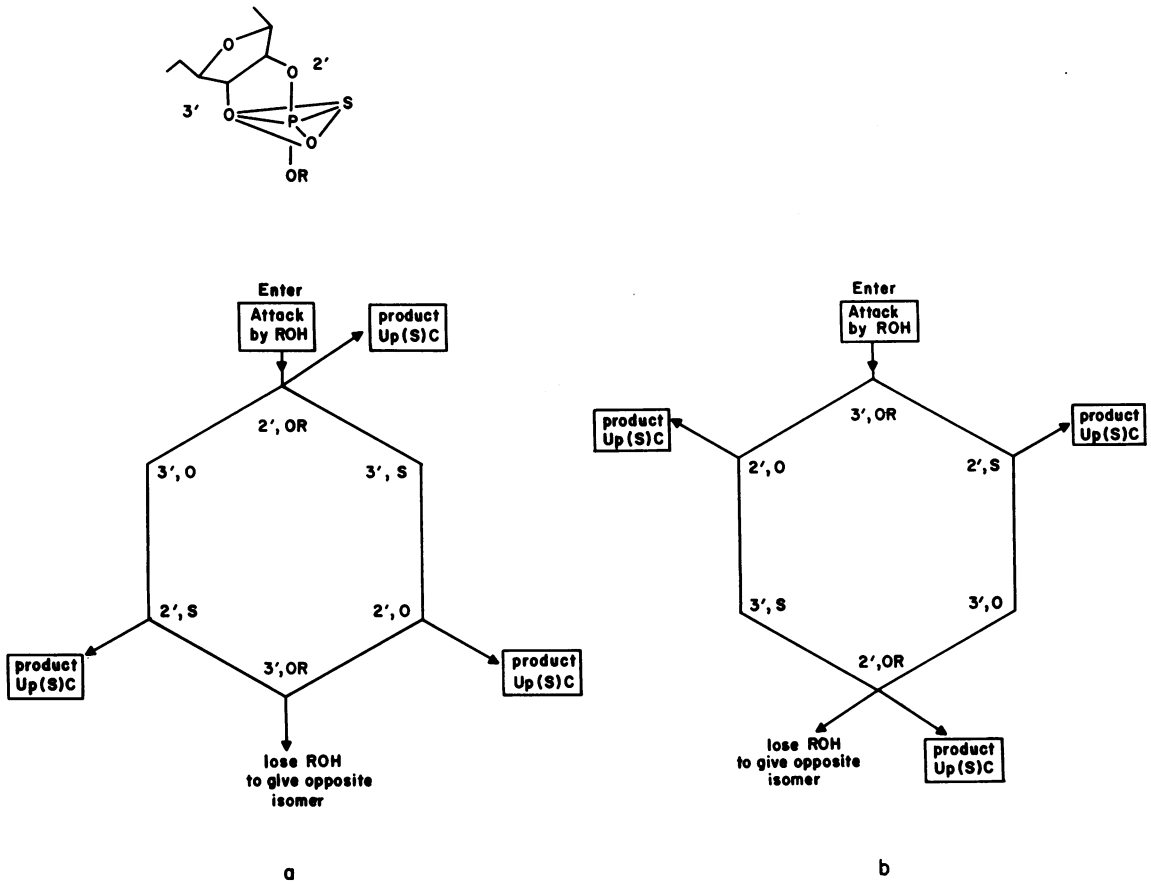

FIG. 4. A pseudorotation diagram for the in-line (*a*) and adjacent (*b*) mechanisms for the attack of the 5′-hydroxyl group of cytidine on uridine 2′,3′-cyclic phosphorothioate [Up̂(S)]. In the in-line case, there is no necessity for an intermediate; the product could form in a concerted displacement. The apexes of the hexagon represent individual pseudorotamers, the structures of which are denoted by the apical groups. The structure shown is, thus, 2′, OR. ROH = Cytidine.

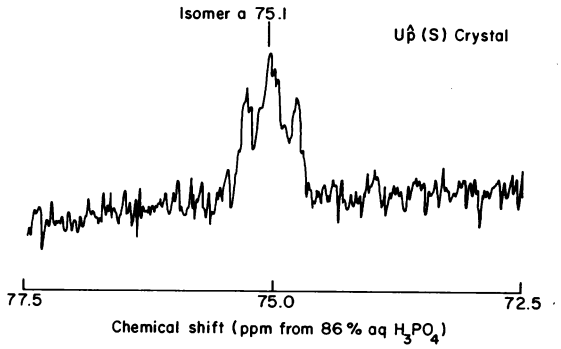

FIG. 5. Fourier-transform ^{31}P NMR spectrum of the crystalline isomer (isomer *a*) of uridine 2′,3′-cyclic phosphorothioate. Chemical shifts given in ppm from 86% (w/w) phosphoric acid in water.

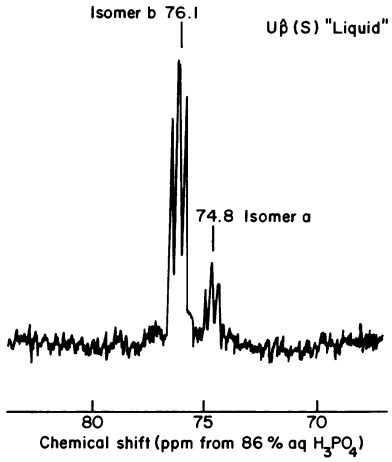

FIG. 6. ^{31}P NMR spectrum of a mixture of the isomers of uridine 2′,3′-cyclic phosphorothioate, containing largely liquid isomer (isomer *b*). Chemical shifts are as in Fig. 5.

clease action, first demonstrated by Heppel, Whitfeld, and Markham (15), was used to synthesize the dinucleoside phosphorothioate Up(S)C from the crystalline isomer of Up̂(S) and cytidine. The stereochemistry of the groups about phosphorus in the product was thus fixed by the choice of isomer and by the geometry of the reaction. This dinucleoside phosphate product was then treated with base to reform cyclic phosphorothioate, but by a nonenzymic reaction that was unequivocally in-line (11). The geometry of the ring opening could then be determined from a comparison of the Up̂(S) product with the Up̂(S) originally used. If the product were again pure crystalline isomer, then the enzymic ring opening was in-line, whereas if the product were the opposite isomer, then the ring opening was adjacent. The geometry of the first step (the transesterification reaction to give cyclic phosphate) could then be determined by invoking the principle of microscopic reversibility.

Enzymic ring opening

The nucleoside can approach phosphorus from either of two directions (Fig. 3). If it attacks from the left (in-line), then the 2′-oxygen assumes an apical position in the resulting trigonal bipyramid and, hence, the P-2′O bond could be cleaved directly (Fig. 4*a*). On the other hand, if attack is from the right (adjacent), then it is the 3′—O that lies apically, requiring at least one pseudorotation to place the 2′—O in an apical position (Fig. 4*b*).

Because of the high hydrolytic activity of the enzyme, only small yields of dinucleoside phosphate product can be isolated unless low temperatures and reasonably high concentrations of reactants, especially the nucleoside, are used. Ustyuzhanin and Kogan (16) were the first to demonstrate a marked increase in yields when the reaction is carried out in frozen solution.

It is essential to establish that it is specifically the (3′–5′) phosphodiester linkage that is formed under the conditions of the synthetic experiment. Any conclusions as to the mechanism of RNase action may be invalid were any (2′–5′) material synthesized, since it is only the (3′–5′) dinucleoside that is the natural substrate for the first step of RNase action (17). Podder and Tinoco (18) have reported that with high concentrations of enzyme and substrate RNase T_1 catalyzes the formation of the (2′–5′) linkage, although Rowe and Smith (19) were subsequently able to obtain entirely (3′–5′) material under conditions that differed somewhat from those of Podder and Tinoco.

To determine the nature of the bond formed under these experimental conditions, we incubated chromatographically

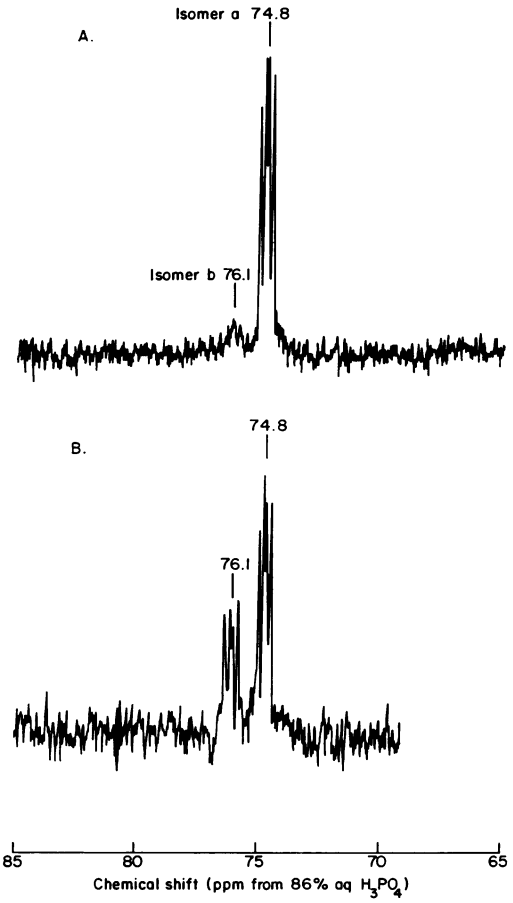

FIG. 7. (*A*) ^{31}P NMR spectrum of the final product of the reaction scheme shown in Fig. 2. Chemical shifts are as in Fig. 5. (*B*) Spectrum of the product in *A* after addition of liquid isomer to the sample tube. Chemical shifts are as in Fig. 5.

pure Up(S)C with the enzyme at room temperature under the usual conditions of dilution. After 2.5 days, the dinucleoside phosphate was completely converted to Up̂(S) and cytidine, as determined by TLE and TLC. Since RNase-A is known to usually hydrolyze exclusively the (3′–5′) linkage of a dinucleoside phosphate, the assumption was made that this was also the case for the related thiophosphate, and it was concluded that only the (3′–5′) isomer was produced during the synthetic reaction.

Base-catalyzed ring closure

In the base-catalyzed reaction of a dinucleoside monophosphate in aqueous solution, the rate of ring closure is not much faster than that of the subsequent hydrolytic ring-opening. Thus, only a small recovery of the intermediate cyclic phosphate is possible. In the present study, therefore, the ring closure was done in tertiary butanol–formamide, with potassium *tert*-butoxide as the base (20). In this way a high yield of the cyclic phosphate can be obtained, since the butoxide is too sterically hindered to act as a nucleophile towards phosphorus. We do not consider it likely that this strong base caused interconversion of the isomers of Up̂(S), since the isomeric purity of the product was as high as that of the starting material, despite some evidence from the preparation of Up̂(S) that the thermodynamic equilibrium gives about 50% of each isomer. A possible path for interconversion could have been via the cyclonucleoside, although we considered this unlikely.

The product

The identity of the Up̂(S) product was determined by ^{31}P NMR. Because of the small amount of material available, the Fourier-transform technique was used, and gave a remarkably clear result. The product was the crystalline isomer, containing less than 5% of the liquid isomer (Figs. 5–7).‡

In the enzymic ring-opening, the reaction was left for a sufficient length of time that an approximately steady state in the concentration of Up(S)C was achieved, and the reaction in both directions

$$\text{Up̂(S)} + \text{C} \rightleftharpoons \text{Up(S)C}$$

must, therefore, have been stereochemically very clean. We conclude that the first step of RNase-A action with Up(S)C is unambiguously in line§, and, as predicted from the x-ray and NMR evidence, the reaction of a normal dinucleoside phosphate substrate may reasonably be inferred to follow the same geometry.

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‡ ^{31}P NMR indicated about 5% contamination by liquid isomer. The starting material itself was only about 95% pure. We considered the possibility that the liquid and crystalline isomers in the original reaction mixture may have reacted with the nucleoside at different rates or with different affinities. Eckstein (21) showed that in the enzymatic hydrolysis of Up̂(S), the two isomers have the same k_2, but K_m for the liquid isomer is eight times higher than for the crystalline isomer. The yield of product Up̂(S) was about 10%, based on the Up̂(S) originally used. Thus, the result is not due to preferential reaction of the impurity in the starting material.

§ Strictly, as with the second step, it is consistent with the pseudorotation diagram that the other isomer of Up̂(S) could react by the adjacent mechanism (Fig. 4*a*), but since the crystalline isomer is nearly as good a substrate as Up̂ (and superior to the liquid isomer), it is most likely that the reaction of the normal phosphate is also in-line.