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Absolute configuration of the diastereomers of adenosine 5′-*O*-(1-thiotriphosphate): Consequences for the stereochemistry of polymerization by DNA-dependent RNA polymerase from *Escherichia coli*

[uridyl-(3′-5′)adenyl-*O,O*-phosphorothioate/high-performance liquid chromatography]

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ABSTRACT **The diastereomers of uridyl-(3′-5′)adenyl-*O,O*-phosphorothioate [Up(S)A] have been separated by high-performance liquid chromatography. Their identification as R_p and S_p follows from the RNase A digestion of these products. It was then shown, by the same method, that the *R* isomer is hydrolyzed by snake venom phosphodiesterase (PDEase) approximately 500 times faster than the *S* isomer. Similarly, the stereoisomer of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS), until now arbitrarily designated as isomer *B*, is hydrolyzed *ca* 400 times faster by PDEase than is isomer A. From these results it is concluded that the *R* isomers of Up(S)A and ATPαS, isomers B, have the same absolute configuration. It then follows that isomer A of ATPαS, the preferred of the two isomers as substrate for DNA-dependent RNA polymerase, has the *S* configuration. The implications for the stereochemistry of action of the latter enzyme are discussed.**

A particularly important piece of information for the description of the mechanism of an enzyme reaction is the stereochemical course of the reaction. The stereochemistry of enzymatic reactions involving carbon has been described for many enzymes by a number of elegant studies (1, 2). For enzymatic reactions at phosphorus, no such studies have been possible until recently because of the lack of chiral phosphate. Nucleotide analogs with a chiral phosphorus, however, became available with the development of nucleoside phosphorothioate esters (3). One such analog, uridine 2′,3′-cyclic *O,O*-phosphorothioate, has been used to establish the stereochemistry of action of RNase A (4–6). Recently, we described the synthesis of the diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) (Fig. 1), one of which was a substrate for DNA-dependent RNA polymerase (7, 8). Although we could determine the absolute configuration of the phosphorothioate diester produced in this reaction, we could not deduce whether this reaction had proceeded with retention or inversion of configuration because we did not know the absolute configuration of diastereomer A of ATPαS, the starting material of this reaction.

We describe here the determination of the absolute configuration of the diastereomers of ATPαS. We show that the diastereomer of uridyl-(3′-5′)adenyl-*O,O*-phosphorothioate [Up(S)A] with the *R* configuration (Fig. 2) is the preferred substrate for snake venom phosphodiesterase (PDEase), as is diastereomer *B* of ATPαS. Correlating these two results leads to the assignment of the *R* configuration to this diastereomer of ATPαS.

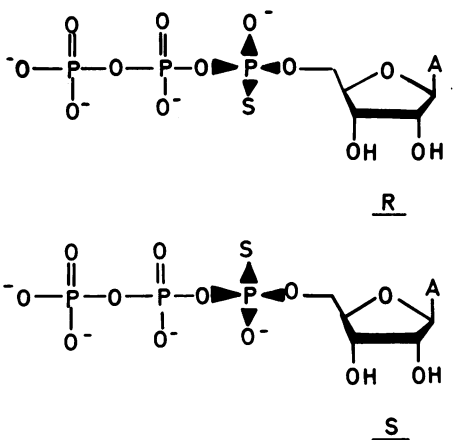

FIG. 1. Diastereomers of ATPαS.

MATERIALS AND METHODS

Materials. Diastereomers A and B of ATPαS were synthesized as described (7). The synthesis of the mixture of diastereomers of Up(S)A will be published elsewhere (9). Pancreatic RNase A (crystalline) and snake venom PDEase (1 mg/ml, specific activity 1.5 units/mg) were obtained from Boehringer Mannheim.

Methods. High-performance liquid chromatography (HPLC) was performed on a Packard-Becker 8200 chromatograph equipped with a Packard 1170 UV detector operating at 254 nm. The strong anion exchanger Nucleosil 10SB from Machery and Nagel (Düren, Germany) was packed into a stainless steel column (50 cm × 1.8 mm) by the slurry method. Elution of the column was effected with buffer A (0.05 M KH_2PO_4/0.25 M KCl, pH 4.5) with a flow rate of 0.62 ml/min at a pressure of 200 atm (1 atm = 100 kPa). The nucleoside triphosphates and their enzymic digestion products were analyzed on a smaller column (40 cm × 2 mm) of Nucleosil 10SB with buffer B (0.05 M KH_2PO_4/0.5 M KCl, pH 4.5) at a flow rate of 1 ml/min and a pressure of 160 atm.

RNase Digestion of Up(S)A (Mixture of Isomers). The reaction solution contained, in a total volume of 50 μl, 3.5 mM Up(S)A (mixture of isomers), 50 mM Tris·HCl (pH 8.2), and 1 μg of RNase A. The reaction course was followed by HPLC. The analysis obtained after 15 min at 25° is presented in Fig. 3*B*.

Preparation of the *S* Isomer of Up(S)A. This stereoisomer was prepared by carrying out the above RNase reaction on a

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Abbreviations: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); Up(S)A, uridyl-(3′-5′)adenyl-*O,O*-phosphorothioate; U>pS, uridine 2′,3′-cyclic *O,O*-phosphorothioate; HPLC, high-performance liquid chromatography; PDEase, snake venom phosphodiesterase.

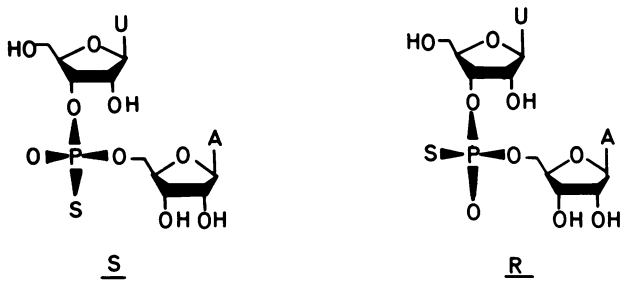

FIG. 2. Diastereomers of Up(S)A.

5-ml scale. The reaction was checked by HPLC for the total disappearance of Up(S)A (*R* isomer) and the mixture then was chromatographed over a column of DEAE-Sephadex A 25, with a linear gradient of 300 ml each of 0.05 M and 0.25 M triethylammonium bicarbonate (pH 7.5).

Enzymatic Digestions with Snake Venom PDEase. All solutions contained, in a total volume of 50 μl, 100 mM Tris·HCl (pH 8.75), 2 mM $MgCl_2$, 2 mM dithiothreitol, 0.2–10 μg of PDE, and the concentrations of dinucleoside monophosphates and nucleoside triphosphates as given in Table 1. The solutions were incubated at 37°. At appropriate times, 10-μl aliquots were quenched with 1 μl of 1 M HCl and analyzed by HPLC.

RESULTS AND DISCUSSION

The chemical synthesis of Up(S)A yields a mixture of the two diastereomers in a ratio of 61:39, as can be derived from their separation of HPLC (9) (Fig. 3*A*; only peaks 6 and 7 are present in this preparation). Incubation of this mixture with pancreatic RNase A leads to the preferred hydrolysis of the predominant diastereomer with concomitant formation of uridine 2′,3′-cyclic *O,O*-phosphorothioate (U>pS) (endo isomer) and adenosine in the expected ratio (Fig. 3*B*). From previous work we know that transesterification of the endo isomer of U>pS results in

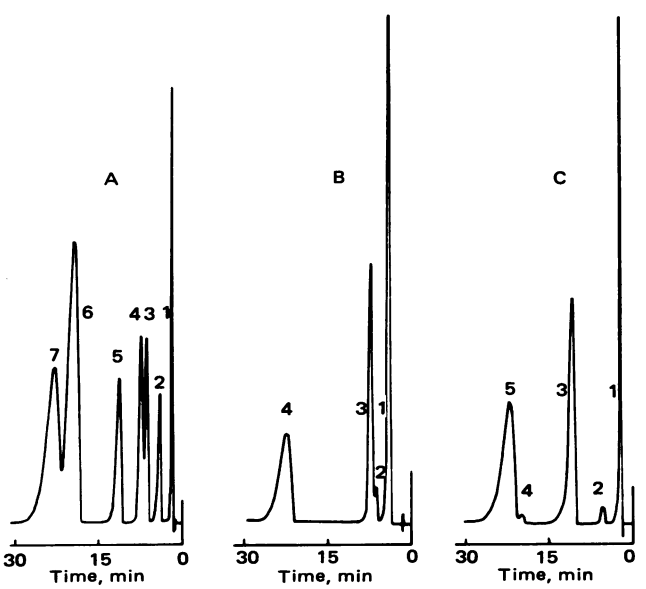

FIG. 3. HPLC analysis of the diastereomers of Up(S)A and their enzymatic digests. (*A*) Separation of the digestion products relevant in this study. Peaks: 1, uridine; 2, adenosine; 3, U>pS (exo); 4, U>pS (endo); 5, adenosine 5′-*O*-phosphorothioate; 6, Up(S)A (*R*); 7, Up(S)A (*S*). (*B*) Partial RNase digestion of Up(S)A (mixture of isomers). Peaks: 1, adenosine; 2, U>pS (exo); 3, U>pS (endo); 4, Up(S)A (*S*). (*C*) Partial PDEase digestion of Up(S)A (mixture of isomers). Peaks: 1, uridine; 2, adenosine 5′-phosphate; 3, adenosine 5′-*O*-phosphorothioate; 4, Up(S)A (*R*); 5, Up(S)A (*S*).

Table 1. Initial rates of PDEase-catalyzed hydrolysis of dinucleotides and nucleoside triphosphates

Substrate	Conc., mM	*V*, nmol/mg protein per min
UpA	5.5	7×10^3
Up(S)A (*S* isomer)	1.5	3.2
Up(S)A (*R* isomer)*	4.2	1.5×10^3
ATP	4.0	14×10^3
ATPαS (isomer A)	4.3	0.8
ATPαS (isomer B)	5.2	340

* The reaction solution also contained 2.8 mM Up(S)A (*S* isomer).

the formation of the internucleotide phosphorothioate linkage with the *R* configuration and it thus follows that hydrolysis of such an isomer with the *R* configuration leads to formation of the endo isomer of U>pS (6, 10). On the basis of this we can assign the *R* configuration to the peak of Up(S)A with the shorter retention time. Prolonged incubation of Up(S)A with RNase also hydrolyzed the diastereomer with the *S* configuration, with formation of the exo isomer of U>pS and adenosine. Snake venom PDEase catalyzes the hydrolysis of an esterified nucleoside 5′-phosphate to the nucleoside 5′-phosphate and alcohol. Its catalytic activity is nonspecific for the alcohol which can be a nucleoside or a phenol and also can be a phosphate or a pyrophosphate—i.e., it hydrolyzes dinucleoside (3′-5′)monophosphates as well as nucleoside 5′-di- and 5′-triphosphates (11, 12). This property of PDEase prompted us to investigate the hydrolysis of the diastereomers of ATPαS as well as Up(S)A by this enzyme in the hope that a comparison of the kinetic data would give us a correlation between the absolute configurations of the diastereomers of ATPαS as well as Up(S)A.

After incubation of the mixture of diastereomers of Up(S)A with PDEase, HPLC showed that only the peak due to the diastereomer with the *R* configuration disappeared, uridine and adenosine 5′-phosphorothioate being formed in the ratio of 1.00:0.95 (Fig. 3*C*). PDEase also slowly desulfurized the adenosine 5′-phosphorothioate formed to give adenosine 5′-phosphate (Fig. 3*C*). However, when the initial stage of the

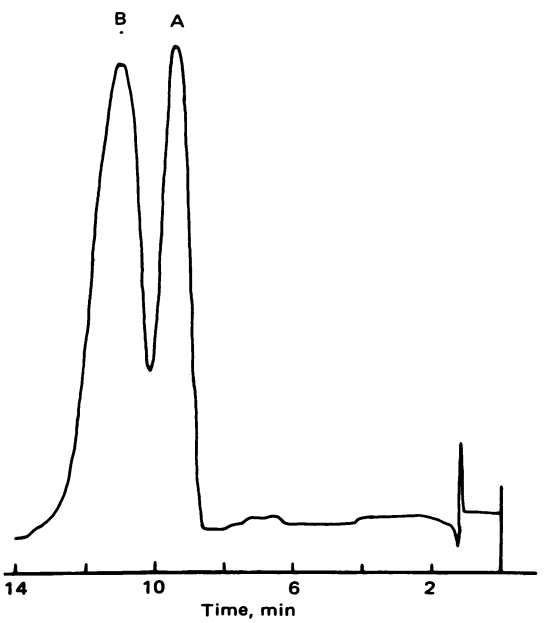

FIG. 4. HPLC separation of ATPαS A and B.

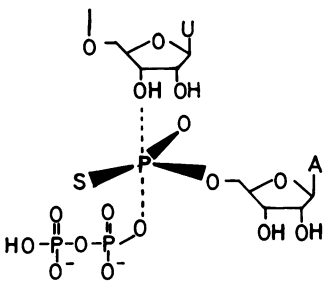

FIG. 5. Trigonal bipyramidal orientation of substrates during RNA-polymerase action.

reaction (less than 10% hydrolysis) was measured, this desulfurization was not observed. The rate difference for hydrolysis of the *R* and *S* diastereomers is about 500-fold (Table 1). This result establishes that the preferred substrate for this enzyme has the *R* configuration. Diastereomers A and B of ATPαS could be separated by HPLC (Fig. 4), providing a convenient and accurate check for their (chiral) purity. Incubation of the A and B isomers of ATPαS with PDEase revealed that only the B isomer was hydrolyzed at an appreciable rate. The rate difference between the two isomers is again several hundred-fold (Table 1), as measured by HPLC.

From these experiments it follows that Up(S)A (*R* isomer) and ATPαS (isomer B) must have the same absolute configuration around phosphorus—i.e., the B isomer of ATPαS also has the *R* configuration. The A isomer is then of the *S* configuration. This, of course, also applies to the diastereomers of ADPαS.

With this information one is in a position to interpret the experimental results reported in our previous publication (8). Polymerization of UTP and the A isomer of ATPαS with *Escherchia coli* DNA-dependent RNA polymerase on poly[d(A-T)] as template resulted in the formation of an alternating polymer in which the phosphorothioate internucleotidic linkage, Up(S)A, had the *R* configuration. Because the A isomer of ATPαS has the *S* configuration, it follows that this enzyme has formed the phosphorothioate linkage with inversion of configuration at the α-phosphorus. The simplest interpretation of this result is that the reaction proceeded by an SN_2 mechanism in an in-line fashion as described (8). At the active site, the 3′-OH group of the growing RNA chain and the pyrophosphate group must thus be positioned in relation to the α-phosphorus of ATPαS in such a way that both can occupy the apical positions of a trigonal bipyramid (Fig. 5). tRNA-Nucleotidyltransferase shows the same stereochemical relationship between substrate and product and therefore the same interpretation of the mechanism applies (13).

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