Stereochemistry of the Transesterification Step of Ribonuclease T_1

Fritz Eckstein,* Hans H. Schulz, Heinrich Rüterjans, Wolfgang Haar, and Wolfgang Maurer

ABSTRACT: The synthesis of the mixture of the two diastereomers of guanosine 2',3'-cyclophosphorothioate is described. Their chemical shifts in the ^{31}P nuclear magnetic resonance (nmr) are identical with those of the corresponding isomers of uridine 2',3'-cyclophosphorothioate (δ −75.22 ppm for the endo, δ −76.75 ppm for the exo isomer). From the unseparable mixture of isomers of guanosine 2',3'-cyclophosphorothioate only the endo isomer is hydrolyzed by ribonuclease T_1 to guanosine 3'-phosphorothioate, without loss of sulfur. The resistant exo isomer is a competitive inhibitor (K_i = 2.5 × 10^{-4} M) for this enzyme. On enzymatic methanolysis of the mixture of isomers only the endo isomer reacts to guanosine 3'-phosphorothioate *O*-methyl ester. The chemical shift of this compound in the ^{31}P nmr (δ −57.55 ppm) is identical with that of uridine 3'-phosphorothioate *O*-methyl ester (*R* configuration) obtained by incubation of the endo isomer of uridine 2',3'-cyclophosphorothioate with pancreatic ribonuclease A in aqueous methanol. This isomer is expected from an in-line mechanism for this reaction. The corresponding methyl ester with the *S* configuration has a δ value of −57.69 ppm. Based on these data, it is suggested that the transesterification step of ribonuclease T_1 follows an in-line mechanism.

Ribonuclease T_1 hydrolyzes guanosine 3'-phosphate esters in a two-step mechanism to guanosine 3'-phosphate with guanosine 2',3'-cyclophosphate as intermediate. Apart from the specificity for guanosine instead of pyrimidine nucleosides it resembles pancreatic ribonuclease A in the overall mechanism. In contrast to pancreatic ribonuclease A, however, little is known about the details of the mechanism of action of this enzyme.

Uridine 2',3'-cyclophosphorothioate has recently been used for investigations of the stereochemistry of the two reaction steps of pancreatic ribonuclease A (Usher *et al.*, 1970, 1972; Eckstein *et al.*, 1972). In this publication we would like to report on the interaction of the analogous guanosine 2',3'-cyclophosphorothioate with ribonuclease T_1.

Experimental Section

Materials and Methods. RNase T_1 (4550 U/mg) was purchased from Sankyo Ltd. (Japan), $P^{35}SCl_3$ from Radiochemical Center, Amersham (England). 2',3'-*O*-Methoxymethylideneguanosine was prepared according to Griffin *et al.* (1967), guanosine 2',3'-cyclophosphate according to Smith *et al.* (1958), triimidazolyl-1-phosphine sulfide according to Eckstein (1970), uridine 3'-phosphorothioate methyl ester (*R* configuration) according to Eckstein *et al.* (1972).

Paper chromatography was carried out unless stated otherwise on paper 2043b (washed) from Schleicher and Schüll (Germany) by the descending method with system A (isopropyl alcohol–H_2O, 7:3, v/v) or B (2 N ammonia–isopropyl alcohol, 3:7 v/v). For quantitative determinations of compounds, the ultraviolet-active spots were excised and eluted with 5 or 10 ml of water–methanol (1:1, v/v), and the optical density was measured. For determination of specific activities, 1 ml of such a solution was counted with 10 ml of a dioxane-based scintillation solution (Bray, 1960) in a Packard Tri-Carb scintillation spectrometer, Model 3375. Thin-layer chromatography (tlc) on SiO_2 was carried out on plates (DC-Fertigplatten, Kieselgel F_{254}) from Merck (Darmstadt) with system A, on PEI-cellulose sheets (Polygram Cel-300 polyethylenimine, uv for tlc, Machery and Nagel, Germany) in 0.5 M LiCl. Electrophoresis was performed on paper Schleicher and Schüll 2043b (washed) in 0.1 M triethylammonium bicarbonate (pH 7.5) with 40 V/cm for 90 min.

^{31}P nuclear magnetic resonance spectroscopy was carried out with a Bruker Physik HFX-6 nmr spectrometer equipped with a Fourier transform (B-Sc-FFT, with a Nicolet 1074 averaging system and a digital equipment PDP-$^8/_2$ computer device). In order to enhance the sensitivity protons were broad band decoupled. Dilute H_3PO_4 was used as external standard. The spectrum reproduced in Figure 4, however, was recorded with a Perkin-Elmer R10 spectrometer connected with a Northern Scientific HS-544 digital memory oscilloscope. The compounds were used as the triethylammonium salts in water (pH approximately, 6.5).

Optical densities were measured with a Zeiss PMQ II and ultraviolet (uv) spectra were recorded on a Cary 14 spectrophotometer.

Kinetic Experiments. The enzymatic hydrolysis of guanosine 2',3'-cyclophosphorothioate and -cyclophosphate was followed by titration with 0.01 N KOH in the titrator TTT-1a of Radiometer (Copenhagen) equipped with a scale expander PHA-630 T, a glass electrode G-2222c, a calomel electrode K-401 and an autoburet ABU-1a. The consumption of base was recorded on a titrigraph SBR-2c. The temperature was held at 30°.

The enzyme was used as a 9.6 × 10^{-4} M solution in 0.2 M NaCl adjusted to pH 7.0. Substrate and inhibitor were also dissolved in 0.2 M NaCl. Microliter quantities of substrate and, where needed, inhibitor solutions were added to 2 ml of 0.2 M NaCl solution (pH 7.0), and the pH was kept at 7.0 with 0.01 N KOH for 1 hr. The reaction was then started by addi-

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, 34 Göttingen, Germany (F. E.), the Gesellschaft für Molekularbiologische Forschung, 3301 Stöckheim bei Braunschweig, Germany (H. H. S.), and the Institut für Aerobiologie, 5949 Grafschaft, Hochsauerland, Germany (H. R., W. H., and W. M.). *Received May 2, 1972.* Part of this work was the subject of a thesis submitted to the Mathematisch–Naturwissenschaftliche Fakultät Braunschweig, 1971, by H. H. S. This work was in part supported by the Deutsche Forschungsgemeinschaft.

tion of enzyme solution. Initial velocities were derived from the recorded consumption of base and plotted in Lineweaver–Burk plots.

Synthesis. 5′-*O*-ACETYLGUANOSINE. 2′,3′-Methoxymethylideneguanosine (4 g, 12.4 mmoles) was dissolved in dry pyridine (30 ml) and acetic anhydride (4.7 ml, 50 mmoles) added. After stirring for 12 hr at room temperature methanol (30 ml) was added, the solution stirred for a further hour and the solvents were evaporated at 35°. The remaining oil was solidified by repeated evaporation with ethanol. Aqueous formic acid (60%, 30 ml) was added and the resulting red solution evaporated to dryness at 35°. After codistillation with ethanol the residue was dissolved in ethanol (60 ml) at 50°. On slow cooling white crystals were obtained which were washed with ethanol: yield 3.7 g (93%); mp 244–246° dec; $\lambda_{max}^{H_2O}$ 253 nm (ϵ 13,600), $\lambda_{min}^{H_2O}$ 223 nm (ϵ 2850); ^{1}H nmr spectra δ 2.04 ppm (s) ($COCH_3$); thin-layer chromatography (tlc) (SiO_2, system chloroform–methanol, 4:1, v/v) R_F 0.45, R_F (guanosine) 0.15, R_F (2′,3′-methoxymethylideneguanosine) 0.58.

GUANOSINE 2′,3′-CYCLOPHOSPHOROTHIOATE. 5′-*O*-Acetylguanosine (3.7 g, 11.3 mmoles) was added to triimidazolyl-1-phosphine sulfide (9 g, 33.8 mmoles) in dry pyridine (250 ml) and shaken for 12 hr at room temperature. The residue obtained after evaporation of the solvents was left in water (100 ml, brought to pH 8.5 with ammonia) for 2 hr at room temperature. The water was evaporated and the residue taken up in concentrated ammonia (150 ml). After 2 hr the reaction mixture was evaporated to dryness, the residue codistilled with water (three times) and the product was chromatographed on a DEAE-cellulose column (bicarbonate form, 4 × 100 cm). After washing the column exhaustively with water until all the nucleoside had been eluted a linear gradient of water (4 l.) and triethylammonium bicarbonate (0.2 M, 4 l.) was applied. The desired product was eluted at a buffer concentration of approximately 0.09 M. The fractions containing the product were evaporated in the presence of Dowex ion-exchange resin (50W-X8, pyridinium form) and eluted from the exchange resin with water, yield 68,700 A_{252} units (44%). For further purification the material was transformed to the Li salt by passage over an ion-exchange column (Dowex 50-WX8, Li form) and chromatographed on paper (system A). For quantities of 50 mg, paper NM-218 from Machery and Nagel (Düren, Germany) was used. The following data were obtained: paper chromatography (system A) R_F 0.58, R_F (guanosine 2′,3′-cyclophosphate) 0.47; $\lambda_{max}^{H_2O}$ 252 nm (ϵ 13,700), $\lambda_{min}^{H_2O}$ 223 nm (ϵ 3000); electrophoretic mobility (pH 7.5) $R_{G>P}$ 0.96; ^{31}P nmr δ −75.22 and −76.75 ppm.

URIDINE 2′,3′-CYCLOPHOSPHOROTHIOATE. To a solution of 5′-acetyluridine (3.8 g, 13 mmoles) in dry pyridine (100 ml) was added a solution of triimidazolyl-1-phosphine sulfide (4.0 g) in dry pyridine (50 ml). After 12 hr at room temperature the solvent was evaporated, and the residue was taken up in water, left for 1 hr, and evaporated to dryness. The residue was dissolved in concentrated ammonia–water (1:1, v/v) (200 ml) and evaporated after 2 hr. The reaction mixture was chromatographed on a DEAE-cellulose column (4 × 50 cm, bicarbonate form) with a linear gradient of water (3 l.) and 0.125 M triethylammonium bicarbonate (3 l.). The desired product contaminated by some uridine 2′,3′-cyclophosphate was eluted at a buffer concentration of approximately 0.09 M. These fractions were evaporated and repeatedly evaporated with methanol. Crystallization of the residue from ethanol afforded the crystalline endo isomer (340 mg). A second crop of crystals was obtained from the mother liquor (210 mg): total 550 mg (9%), mp 204–206°, ^{31}P nmr δ −75.22 ppm.[1] On purification of the mother liquor by paper chromatography (system B), the exo isomer could be obtained (8000 A_{260} units, 6%): ^{31}P nmr δ −76.75 ppm.

RESISTANT ISOMER OF GUANOSINE 2′,3′-CYCLOPHOSPHOROTHIOATE. Guanosine 2′,3′-cyclophosphorothioate (mixture of isomers, Li salt, 1500 A_{252} units) was dissolved in 0.2 M NaCl (2 ml, pH 7.0) in the titration vessel of the autoburet and brought to pH 7.0 and a solution (1 ml) of ribonuclease T_1 in 0.2 M NaCl (pH 7.0, 2 mg of enzyme/ml) was added. After stirring for 11 hr at 30° and keeping the pH at 7.0 with 0.5 N KOH the calculated amount of base for 50% hydrolysis was consumed. Separation of the reaction products after concentration by evaporation was achieved by paper chromatography (system B): yield of guanosine 2′,3′-cyclophosphorothioate (resistant isomer) 630 A_{252} units (42%).

GUANOSINE 2′(3′)-PHOSPHOROTHIOATE. Guanosine 2′,3′-cyclophosphorothioate (100 A_{252} units) was dissolved in water (0.18 ml), and 0.4 N KOH (0.30 ml) was added, left at room temperature for 6 hr, and neutralized by addition of 60% aqueous $HClO_4$ (approximately 5 μl). Purification by paper chromatography (system B) yielded 97 A_{252} units of guanosine 2′(3′)-phosphorothioate. Using [^{35}S]guanosine 2′,3′-cyclophosphorothioate (85 A_{252} units, in 0.3 ml of water, 216,512 cpm/A_{252} unit) as substrate, the reaction was stopped after 1 hr. An aliquot of the reaction (200 μl) was chromatographed on paper. [^{35}S]Guanosine 2′(3′)-phosphorothioate (16 A_{252} units, 56%) with 215,073 cpm/A_{252} units was thus isolated.

HYDROLYSIS OF GUANOSINE 2′,3′-CYCLOPHOSPHOROTHIOATE BY RIBONUCLEASE T_1. Ratio of Products. Guanosine 2′,3′-cyclophosphorothioate (17 A_{252} units) was dissolved in 0.1 M Tris-HCl (pH 7.6, 60 μl), and x μl of ribonuclease T_1 solution (10 mg/ml) and 50 − x μl of water were added. After 4 hr at 37° the reaction mixture was separated by paper chromatography (system B) and the amount of guanosine 3′-phosphorothioate and guanosine 2′,3′-cyclophosphoreothioate was determined. With 10 μl of enzyme solution 8.60 A_{252} units of guanosine 2′,3′-cyclophosphorothioate and 7.92 A_{252} units of guanosine 3′-phosphorothioate were isolated. With 40 μl of enzyme the amounts were 8.73 and 8.16, respectively.

Retention of Sulfur. To [^{35}S]guanosine 2′,3′-cyclophosphorothioate (40 A_{252} units) dissolved in H_2O (10 μl) and 0.1 M Tris-HCl (pH 7.6, 0.10 ml), an aqueous solution of ribonuclease T_1 (11 mg/ml, 20 μl) was added. Immediately after addition of enzyme an aliquot (50 μl) was removed and chromatographed (system B). The rest was chromatographed after 3.5-hr incubation at 37°. [^{35}S]Guanosine 2′,3′-cyclophosphorothioate (8.40 A_{252} units) isolated after 3.5-hr incubation had 129,080 cpm/A_{252} units, [^{35}S]guanosine 3′-phosphorothioate (6.20 A_{252} units) 129,770 cpm/A_{252} units. [^{35}S]-Guanosine 2′,3′-cyclophosphorothioate after 1-min incubation had 134,565 cpm/A_{252} units.

URIDINE 3′-PHOSPHOROTHIOATE *O*-METHYL ESTER (*S* CONFIGURATION). Uridine 2′,3′-cyclophosphorothioate (1700 A_{260} units, noncrystalline isomer, triethylammonium salt) was dissolved in 0.1 M ethylenediamine buffer (pH 7.0, 1 ml)–methanol (8 ml) and an aqueous solution of pancreatic ribonuclease A (1 ml, 30 mg/ml) was added. A precipitate was formed and after 4 hr at room temperature 1 M ethylenediamine (0.3 ml) was added, the solution was evaporated to

[1] The chemical shifts for the two isomers of uridine 2′,3′-cyclophosphorothioate reported earlier (Eckstein, 1970) have to be corrected by +10% due to a compression of the spectra by the digital memory oscilloscope unknown at the time of the recording.

TABLE I: Chromatography of Guanosine Phosphates and Phosphorothioates.

Compound	PEI-Cellulose[a]	R_F system A[b]	R_F system B[b]
Guanosine 3′-phosphate	0.25	0.40	0.09
Guanosine 2′,3′-cyclophosphate	0.66	0.47	0.20
Guanosine 3′-phosphate methyl ester	0.83		
Guanosine 3′-phosphorothioate	0.16	0.43	0.45
Guanosine 2′,3′-cyclophosphorothioate	0.46	0.54	0.45
Guanosine 3′-phosphorothioate methyl ester	0.66		

[a] With 0.5 M LiCl. [b] Paper chromatography.

dryness, the residue was taken up in water and the three detectable compounds were isolated by preparative paper chromatography (system B). Uridine 3′-phosphorothioate (140 A_{260} units, R_F 0.36), uridine 2′,3′-cyclophosphorothioate (520 A_{260} units, R_F 0.59), and uridine 3′-phosphorothioate *O*-methyl ester (480 A_{260} units, R_F 0.67) were obtained. After transformation into the triethylammonium salt this methyl ester could not be crystallized: ^{31}P nmr δ −57.69 ppm.

GUANOSINE 3′-PHOSPHOROTHIOATE *O*-METHYL ESTER. To a solution of guanosine 2′,3′-cyclophosphorothioate (180 A_{252} units) in water (0.60 ml)–1 M Tris-HCl (pH 7.6, 15 μl)–methanol (0.75 ml) was added an aqueous solution of ribonuclease T_1 (0.15 ml, 10 mg/ml). After 3 hr at room temperature 0.1 M dithiothreitol (75 μl) was added and the solution was chromatographed on a QAE A-25 Sephadex column (1.5 × 14 cm, bicarbonate form) with a linear gradient of 250 ml each of 0.05 and 0.25 M triethylammonium bicarbonate. Guanosine 3′-phosphorothioate *O*-methyl ester (34 A_{252} units) and guanosine 2′,3′-cyclophosphorothioate (40 A_{252} units) could be isolated and identified by tlc on PEI-cellulose[2] (Table I). ^{31}P nmr spectroscopy of the methyl ester showed a δ value of −57.55 ppm.

HYDROLYSIS OF GUANOSINE 3′-PHOSPHOROTHIOATE *O*-METHYL ESTER. To a solution of guanosine 3′-phosphorothioate *O*-methyl ester (8 A_{252} units) in water (10 μl) and 0.1 M Tris-HCl (pH 7.6, 50 μl) was added an aqueous solution of ribonuclease T_1 (10 μl, 10 mg/ml). After incubation at 37° for 1.5 hr tlc on PEI-cellulose revealed guanosine 3′-phosphorothioate as the sole product.

Results

Synthesis. As reported earlier (Eckstein and Gindl, 1968) uridine 2′,3′-cyclophosphorothioate can be synthesized from 5′-acetyluridine and triimidazolyl-1-phosphine sulfide. We report here a slightly modified procedure and the separation of the two diastereomers by crystallization. The assignment of the endo configuration to the crystalline isomer (δ −75.22 ppm) by X-ray structure analysis was reported elsewhere (Saenger and Eckstein, 1970).

[2] PEI-cellulose is polyethylenimine cellulose.

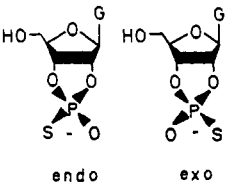

FIGURE 1: Diastereomers of guanosine 2′,3′-cyclophosphorothioate.

Guanosine 2′,3′-cyclophosphorothioate could be synthesized analogously by reaction of 5′-acetylguanosine with triimidazolyl-1-phosphine sulfide. As shown by ^{31}P nmr spectroscopy (Figures 1 and 2), two diastereomers are also found in this case. In contrast to uridine 2′,3′-cyclophosphorothioate the isomers of guanosine 2′,3′-cyclophosphorothioate could not be separated by crystallization. The chemical shifts of the mixture of isomers for both nucleotides are identical. We therefore feel confident that the same assignment of configuration to the two signals applies in both cases. Thus, the signal with a δ value of −75.22 ppm is due to the endo isomer guanosine 2′,3′-cyclophosphorothioate as is the case with the corresponding uridine compound.

Alkaline hydrolysis of the mixture of isomers of guanosine 2′,3′-cyclophosphorothioate led to the formation of guanosine 2′(3′)-phosphorothioate. The results with [^{35}S]guanosine 2′,3′-cyclophosphorothioate show that no sulfur is lost in this reaction as has also been shown for the alkaline hydrolysis of [^{35}S]uridine 2′,3′-cyclophosphorothioate (Eckstein and Gindl, 1968).

Enzymatic Hydrolysis. On incubation of the mixture of isomers of guanosine 2′,3′-cyclophosphorothioate with ribonuclease T_1 not more than 50% could be hydrolyzed even with a large amount of enzyme. When this hydrolysis was followed by ^{31}P nmr spectroscopy, only disappearance of the signal for the endo isomer could be detected (Figure 3). The resistant exo isomer could be isolated from such a reaction by paper chromatography and its interaction with the enzyme could be studied separately. The exo isomer is a competitive inhibitor (K_i = 2.5 × 10^{-4} M) of the hydrolysis of guanosine 2′,3′-cyclophosphate (K_m = 5 × 10^{-4} M, V_{max} = 4.0 μmole min^{-1} mg^{-1}) (Figure 4). Irie (1967) reported K_m (GcP) = 1.5 × 10^{-3} M for slightly different conditions.

The initial velocities measured for the hydrolysis of the 1:1 mixture of isomers were not accurate enought to determine K_m and V_{max} values. With [^{35}S]guanosine 2′,3′-cyclophosphorothioate as substrate no loss of sulfur could be observed in the enzymatic hydrolysis. The limit of error in the

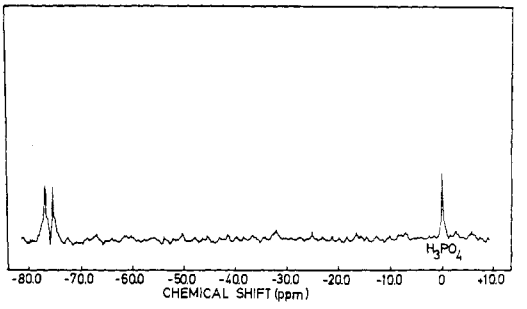

FIGURE 2: ^{31}P nmr spectrum of mixture of diastereomers of guanosine 2′,3′-cyclophosphorothioate (10 mg/2 ml of D_2O); temperature 27°; δ −76.76 ± 0.06 and −75.22 ± 0.06 ppm. Pulse Fourier transform spectrum of 4000 pulses.

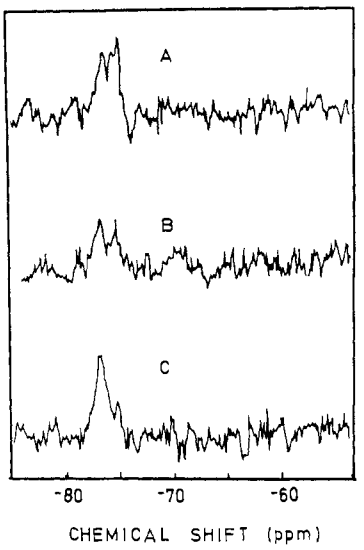

FIGURE 3: Change of ^{31}P nmr spectrum of mixture of diastereomers of guanosine 2′,3′-cyclophosphorothioate upon enzymatic hydrolysis. Mixture of isomers (0.5 mmole) dissolved in 3.0 ml of 0.05 M Tris-HCl (pH 7.5) (A); incubation with ribonuclease T_1 (400 μl, 4 mg/ml) for 21 hr at 30° (B); after addition of another 100 μl of enzyme (10 mg/ml) and incubation for 6 hr (C).

method applied is estimated to be around 5% mainly because last traces of contaminating radioactive sulfur are difficult to remove from the starting material even by repeated chromatography.

Guanosine 2′(3′)-phosphorothioate obtained from alkaline hydrolysis of guanosine 2′,3′-cyclophosphorothioate is a competitive inhibitor, $K_i = 3.8 \times 10^{-5}$ M (Figure 4). The value reported (Irie, 1964) for 3′-GMP is $K_i = 6.6 \times 10^{-5}$ M, for 2′-GMP $K_i = 3.4 \times 10^{-5}$ M. In another publication (Irie, 1967) the latter is reported as 1.7×10^{-4} for slightly different conditions.

Enzymatic Transesterification. On incubation of the mixture of isomers of guanosine 2′,3′-cyclophosphorothioate in 50% aqueous methanol with ribonuclease T_1 guanosine 3′-phosphorothioate *O*-methyl ester was formed in about 20% yield. ^{31}P nmr spectroscopy of the reisolated unreacted guanosine 2′,3′-cyclophosphorothioate (Figure 5) reveals that it is the

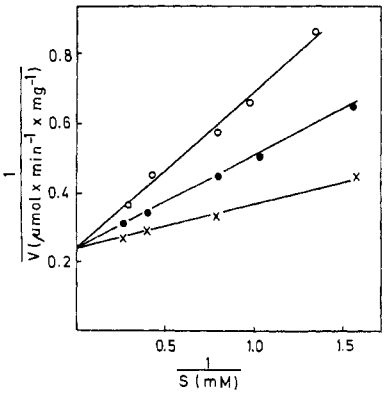

FIGURE 4: Lineweaver–Burk plot of hydrolysis of guanosine 2′,3′-cyclophosphate. In absence of inhibitor (×); in the presence of guanosine 2′(3′)-phosphorothioate (4.4×10^{-5} M) (○); in the presence of exoisomer of guanosine 2′,3′-cyclophosphorothioate (6.5×10^{-4} M) (●).

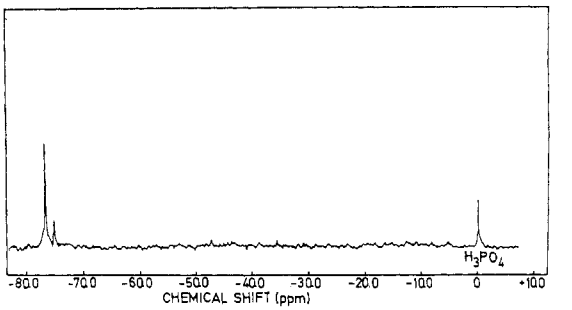

FIGURE 5: ^{31}P nmr spectrum of the same mixture of diastereomers of guanosine 2′,3′-cyclophosphorothioate as in Figure 2 after enzymatic methanolysis. Conditions and chemical shifts identical with those in Figure 2.

exo isomer and thus that, therefore, only the endo isomer had reacted as in the hydrolysis reaction. The chemical shift of the phosphorus resonance of the methyl ester is δ −57.55 ppm which is identical with that of the methyl ester isolated from the reaction of the crystalline endo isomer of uridine 2′,3′-cyclophosphorothioate with methanol and pancreatic ribonuclease A (Figure 6). Hydrolysis of this guanosine 3′-phosphorothioate *O*-methyl ester with ribonuclease T_1 led to guanosine 3′-phosphorothioate with no detectable amount of guanosine 2′,3′-cyclophosphorothioate remaining.

Discussion

Ribonuclease T_1 is a well-characterized, crystalline enzyme whose amino acid sequence is known (Takahashi, 1971b; Uchida and Egami, 1971) and which hydrolyzes ribonucleic acids by cleavage of the guanosine 3′-phosphate ester bonds in a two-step mechanism. The first step is a transesterification reaction to guanosine 2′,3′-cyclophosphate which is hydrolyzed in the second step to guanosine 3′-phosphate. The transesterification step is easily reversible. Incubation of guanosine 2′,3′-cyclophosphate with an alcohol or a nucleoside leads to the formation of a guanosine 3′-phosphate ester. Thus, there exists a great similarity to the overall mechanism of pancreatic ribonuclease. As reviewed by Richards and Wyckoff (1971) recently for pancreatic ribonuclease A, both the transesterification as well as the hydrolytic step can principally follow two mechanisms. These have been classified as in-line and adjacent (Usher, 1969) depending on whether

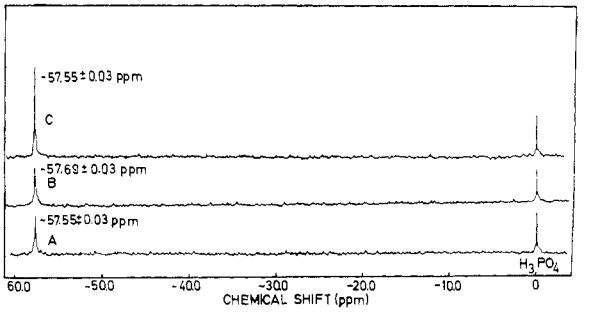

FIGURE 6: Comparison of ^{31}P nmr spectra of nucleoside 3′-phosphorothioate *O*-methyl esters. Uridine 3′-phosphorothioate *O*-methyl ester, *R* configuration (A), *S* configuration (B), and guanosine 3′-phosphorothioate *O*-methyl ester (C). Concentrations 5 mg/2 ml of D_2O; temperature 27°; pulse Fourier transform spectra of 4000 pulses.

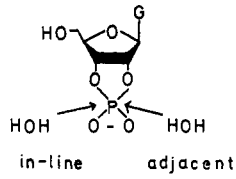

FIGURE 7: Classification of mechanisms for hydrolysis of cyclic phosphates.

the incoming nucleophile attacks the phosphorus on the same or the opposite side as that from which the outgoing group leaves (Figure 7). Both mechanisms can be visualized as proceeding through a pentacoordinated phosphorus intermediate in the form of a trigonal bipyramid as discussed by Westheimer (1968). The adjacent mechanism requires pseudorotation of this intermediate whereas the in-line mechanism does not. Using the endo isomer of uridine 2′,3′-cyclophosphorothioate attempts have been undertaken recently to distinguish between these two mechanisms for pancreatic ribonuclease A. Evidence was obtained which would support an in-line mechanism for both steps (Usher *et al.* 1970, 1972; Eckstein *et al.*, 1972). In analogy with guanosine 2′,3′-cyclophosphorothioate as substrate it should be possible to decide this question for ribonuclease T_1.

On incubation of the mixture of isomers of guanosine 2′,3′-cyclophosphorothioate with ribonuclease T_1 only the nmr signal of the endo isomer disappeared (Figure 3). Thus, only the endo isomer seems to be a substrate for this enzyme. To determine whether the resistance of the exo isomer was due to a lack of binding we carried out inhibition experiments with the reisolated material. As seen from Figure 4 it is a competitive inhibitor with a K_i similar to the K_m of guanosine 2′,3′-cyclophosphate. The reason for the resistance of the exo isomer must therefore lie in a difference in reactivity once the two isomers are bound to the enzyme. It is of interest to note that, in contrast, both isomers of uridine 2′,3′-cyclophosphorothioate are substrates for pancreatic ribonuclease A, differing in the K_m values by a factor of about 8 (Eckstein, 1968).

With [^{35}S]guanosine 2′,3′-cyclophosphorothioate as substrate we found no loss of sulfur during the enzymatic hydrolysis. This result is consistent with an in-line mechanism for the hydrolysis step but does not prove it as discussed by Usher *et al.* (1970) for the hydrolysis of [^{35}S]uridine 2′,3′-cyclophosphorothioate by pancreatic ribonuclease where no sulfur is lost either (Eckstein, 1968).

On replacement of water by methanol in the ring-opening reaction again only the endo isomer of guanosine 2′,3′-cyclophosphorothioate was found to react (Figure 5). The guanosine 3′-phosphorothioate *O*-methyl ester isolated from this reaction has a chemical shift of the phosphorus resonance of δ −57.55 ppm (Figure 6). This shift is identical with that of uridine 3′-phosphorothioate *O*-methyl ester with the *R* configuration obtained by reaction of the endo isomer of uridine 2′,3′-cyclophosphorothioate with pancreatic ribonuclease A as determined by X-ray structure analysis (Eckstein *et al.*, 1972). The chemical shift of the phosphorus resonance of the isomeric uridine 3′-phosphorothioate *O*-methyl ester (*S* configuration) obtained by reaction of the exo isomer of uridine 2′,3′-cyclophosphorothioate with pancreatic ribonuclease A is δ −57.69 ppm (Figure 6).

Although the difference in chemical shift between the two isomeric methyl esters is small, it is fully reproducible. This consistent difference shows that the isomers are stable and do not racemize. The chemical shift (δ −57 ppm) also proves that there is no migration of the methyl group from oxygen to sulfur. For such an *S*-methyl ester a chemical shift of δ −17.0 ppm has to be expected (D. Shire and F. Eckstein, unpublished results).

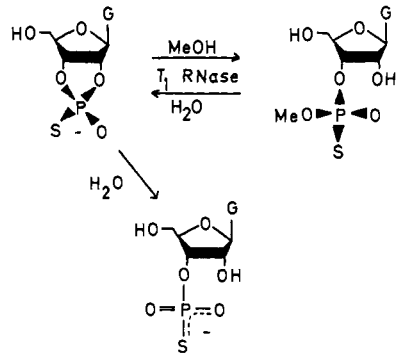

FIGURE 8: Stereochemistry of the transesterification reaction of ribonuclease T_1.

From the identity of the chemical shifts for the uridine 3′-phosphorothioate *O*-methyl ester with the *R* configuration and the guanosine 3′-phosphorothioate *O*-methyl ester we conclude that the latter also has the *R* configuration. Since both compounds have been obtained from the *endo*-cyclophosphorothioate one is lead to conclude that both reactions proceeded by the same stereochemical course. As discussed for the stereochemistry of the reaction of uridine 2′,3′-cyclophosphorothioate with pancreatic ribonuclease A and methanol (Eckstein *et al.*, 1972) the simplest mechanism to explain the production of the uridine 3′-phosphorothioate *O*-methyl ester with the *R* configuration is the in-line mechanism. It follows that the stereochemistry of the transesterification step of ribonuclease T_1 can also most easily be explained by an in-line mechanism (Figure 8).

As mentioned above, the in-line mechanism does not require pseudorotation for product formation. However, two successive pseudorotations will also lead to the same product (Usher *et al.*, 1972). This latter possibility can therefore not be excluded on the basis of the data presented here.

The enzymatic hydrolysis of the guanosine 3′-phosphorothioate *O*-methyl ester by ribonuclease T_1 to guanosine 3′-phosphorothioate apparently occurs *via* the endo isomer of guanosine 2′,3′-cyclophosphorothioate as intermediate. If the exo isomer was the intermediate the reaction would stop at this stage since this isomer is resistant to enzymatic hydrolysis. It follows that the transesterification reaction in both directions in water or aqueous methanol follows the in-line mechanism (Figure 8). There is evidence from photooxidation (Takahashi, 1971a) and ^{1}H nmr studies (Rüterjans and Pongs, 1971) that two histidines, and from carboxymethylation that glutamic acid-58 (Takahashi *et al.*, 1967) are positioned in the active center of ribonuclease T_1. Based on these findings Takahashi (1970) has proposed a mechanism where one histidine and glutamic acid-58 are the proton donor and acceptor, respectively. Although this mechanism is still speculative it is entirely consistent with an in-line mechanism. Alternatives with other amino acids as proton donors and acceptors cannot be excluded at present. Mechanisms with only one functional group involved in catalysis, however, are unlikely since an in-line mechanism requires two such groups.

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A Kinetic Investigation of the Interaction of Serine Transhydroxymethylase with Glycine†

Chao-Fu Cheng and John L. Haslam*

ABSTRACT: Five elementary reactions have been observed by the temperature-jump method in the serine transhydroxymethylase–glycine system. Kinetic experiments were carried out over a large glycine concentration range (0.5–100 mM), and the reactions were monitored at three wavelengths 495, 425, and 343 nm in order to determine the concentration and wavelength dependence of each step. From the analysis of the kinetic data a bimolecular and four unimolecular reactions are indicated. In the analysis, all of the reactions were considered as part of a single linear reaction mechanism, and rate constants for two of the possible linear mechanisms have been determined. The steps in the enzyme–glycine interaction are discussed in relationship to the kinetic results and to the proposed intermediates in pyridoxal-P-dependent enzyme reactions.

The temperature-jump method has been used to investigate many enzyme–substrate reactions. See, for example, Hammes (1968) and Gutfreund (1971). Among the enzymes studied by this method are several pyridoxal-P-dependent enzymes (Fasella and Hammes, 1967; Hammes and Haslam, 1968, 1969; and Faeder and Hammes, 1970, 1971). Kinetic investigations with these enzymes are facilitated because of the visible absorptions associated with pyridoxal-P, which absorption can be used as indicators for the enzyme reactions. On the basis of the various peaks in the spectra of these enzymes with their substrates several different intermediate structures have been postulated, such as a Schiff base (Metzler, 1957), geminal diamine (O'Leary, 1971) and quinoid-type structures (Jenkins, 1964). Schirch and Mason (1963) showed that addition of glycine to a solution of serine transhydroxymethylase (L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1) resulted in a decrease in the enzyme absorption at 425 nm while producing new peaks at 343 and 495 nm. The peak at 425 nm is still observed even when the enzyme is saturated with glycine. These results indicate the formation of three different enzyme-glycine complexes. Schirch and Diller (1971) concluded that a conformational process may also occur in the enzyme–glycine system based on the results of a temperature-dependence study of the absorption changes at 343 and 425 nm. In the work reported here a kinetic study was done to determine the number and nature of elementary reactions involved in the serine transhydroxymethylase–glycine interaction.

Experimental Section

Serine transhydroxymethylase was prepared from fresh iced rabbit livers by the procedure of Schirch and Gross (1968) with the following modifications: the heat step was performed at 63° in the presence of 0.02 M DL-serine, the pH during the first ammonium sulfate precipitation was adjusted to pH 7 by the addition of 1 M potassium hydroxide, the hydroxylapatite column was eliminated, and the enzyme was crystallized by the addition of ammonium sulfate.

Enzyme concentrations were estimated using an extinction coefficient of 0.95 ml/mg cm at 280 nm (Fujioka, 1969). The

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