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Stereochemistry of Internucleotidic Bond Formation by tRNA Nucleotidyltransferase from Baker's Yeast†

Fritz Eckstein,* Hans Sternbach, and Friedrich von der Haar

ABSTRACT: Isomer A of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) is a substrate for tRNA nucleotidyltransferase from baker's yeast, whereas isomer B is a competitive inhibitor. The tRNA resulting from this reaction has a phosphorothioate instead of a phosphate diester linkage at the last internucleotidic linkage between cytidine and adenosine. On limited digestion of this tRNA with RNase A, one can isolate cytidine 2′,3′-cyclic phosphorothioate which can be deaminated to uridine 2′,3′-cyclic phosphorothioate. It can be shown that this compound is the endo isomer and that, therefore, the phosphorothioate diester bond in the tRNA must have had the *R* configuration. This result indicates that no racemization during the condensation of ATPαS, isomer A, onto the tRNA had occurred. Whether inversion or retention of configuration had taken place awaits elucidation of the absolute configuration of isomer A of ATPαS.

Transfer ribonucleic acid nucleotidyltransferase has been isolated from a variety of sources. It catalyzes the incorporation of CMP and AMP residues into the 3′ terminus of tRNAs, taking as substrates CTP, ATP, and tRNAs lacking the CCA end (Deutscher, 1974). The enzyme from baker's yeast has been used to incorporate a number of CTP and ATP analogues into tRNAs and it has been shown to possess an SH group essential for enzymatic activity (Sternbach et al., 1976). Its biological function is not as yet clearly established, although it is conceivable that it is responsible for the completion of the CCA end of tRNAs lacking this sequence partly or totally (Seidman and Mc Clain, 1975).

This enzyme belongs to the class of enzymes which is capable of forming phosphodiester bonds and thus resembles RNA and DNA polymerases. There is little known about the mechanism by which these enzymes condense the terminal 3′-hydroxyl group of a growing polynucleotide chain with the α-phosphate group of a nucleoside 5′-triphosphate. The

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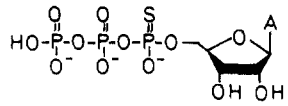

FIGURE 1: Adenosine 5′-*O*-(1-thiotriphosphate).

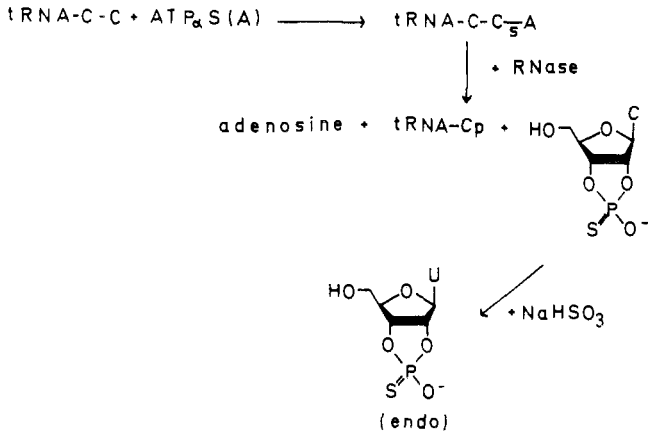

FIGURE 2: Sequence of reactions for the determination of configuration of the phosphorothioate linkage in tRNA-C-C$_s$A.

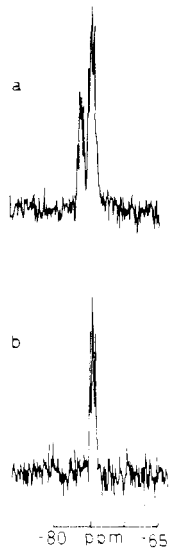

FIGURE 3: ^{31}P NMR spectra. (a) Mixture of authentic crystalline U>pS (endo isomer, δ −74.49 ppm) and noncrystalline U>pS (exo isomer, δ −76.00 ppm). (b) C>pS (δ −74.58 ppm) isolated from tRNA-C-C$_s$A.

availability of an ATP analogue, ATPαS,[1] with a chiral α-phosphorus makes studies of the stereochemical course of this reaction feasible (Figure 1). The diastereomers of ATPαS can be separated (Eckstein and Goody, 1976) and have been used recently for studies of the stereochemistry of polymerization by DNA-dependent RNA polymerase (Eckstein et al., 1976). In this investigation, we have extended this study to the tRNA nucleotidyltransferase (Figure 2).

Materials and Methods

Materials

tRNA^{Phe}-C-C from yeast was prepared from commercial bulk tRNA (Boehringer, Mannheim, Germany) as described (Schneider et al., 1972). Partially purified tRNA-C-C consisting mainly of tRNA^{Ser} (45%) and tRNA^{Tyr} (40%) was obtained by BD-cellulose chromatography as reported (Schneider et al., 1972).

tRNA nucleotidyltransferase (EC 2.7.7.25) from commercial baker's yeast was purified to homogeneity (specific activity 4000 units/mg) by a slight modification of a published procedure (Sternbach et al., 1971). Pancreatic RNase was purchased from Boehringer (Mannheim, Germany).

[^{14}C]ATP (42 Ci/mol) was obtained from Schwarz/Mann (Orangeburg, N.Y.). The diastereomers of [^{35}S]ATPαS were prepared as published (Eckstein and Goody, 1976).

Methods

The assay for AMP incorporation into tRNA^{Phe}-C-C has been described in a previous publication (Sternbach et al., 1976). The incorporation of AMPS was monitored in the same way.

Synthesis of [^{35}S]tRNA-C-C$_s$A. tRNA-C-C (35 000 A_{260} units; ≈55 μmol) was dissolved in 300 mL of incubation mixture containing 0.05 M Tris-HCl, pH 9.0; 0.1 M KCl; 5 mM $MgSO_4$; and 0.16 mM [^{35}S]ATPαS, isomer A. Thus, the tRNA/[^{35}S]ATPαS ratio was roughly 1:1. tRNA nucleotidyltransferase (15 000 units) was added and the solution was incubated at room temperature overnight. The mixture was then adjusted to pH 5.2 by addition of 2 M sodium acetate buffer, pH 4.5. tRNA and ATPαS were adsorbed on a 350-mL Sephadex A-25 column. [^{35}S]ATPαS and buffer constituents were removed by washing the column with 0.5 M NaCl solution. [^{35}S]tRNA-C-C$_s$A was eluted with 1 M NaCl. The tRNA was desalted by passage over a Bio-Gel P2 column (40 × 5 cm) (Bio-Rad, Richmond, Va.) equilibrated with water. The yield was 27 500 A_{260} units of [^{35}S]tRNA-C-C$_s$A with 14 500 cpm/A_{260} unit.

Digestion with RNase. [^{35}S]tRNA-C-C$_s$A (18 000 units) was dissolved in 36 mL of incubation mixture containing 0.15 M Tris-HCl, pH 7.6, and 5 mM $MgSO_4$. After adding 20 μg of RNase, the solution was incubated at room temperature. The reaction was followed by applying 5-μL aliquots to silica-gel plates (Merck, Darmstadt) and developing the plates with benzene/methanol 7:3 (v/v). In this system, [^{35}S]-tRNA-C-C$_s$A and [^{35}S]CMPS have R_f values less than 0.05, whereas [^{35}S]C>pS has a R_f of 0.7–0.8. Pilot experiments were performed in order to determine the optimal amount of RNase required to ensure that digestion to [^{35}S]C>pS was complete without further hydrolysis to [^{35}S]CMPS. After 4 h, the reaction mixture was passed over a Sephadex G-25 column (3 × 35 cm) equilibrated with water. The bulk of the UV-absorbing material was eluted with the break-through volume and did not contain any radioactivity. [^{35}S]C>pS eluted in the late fractions just after the buffer constituents contaminated mainly with adenosine. The yield was 670 A_{271} units containing all the ^{35}S label. This material was applied to a DEAE-Sephadex column (1.5 × 30 cm) and eluted with a linear gradient of 500 mL each of water and 0.2 M triethylammonium bicarbonate. The yield was 300 A_{272} units of [^{35}S]C>pS (16.1 × 10^6 cpm).

[1] Abbreviations used are: ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); CMPS, cytidine 3′-(2′)-phosphorothioate; C>pS, cytidine 2′,3′-cyclic phosphorothioate; U>pS, uridine 2′,3′-cyclic phosphorothioate; tRNA-C-C, tRNA lacking the terminal AMP residue; tRNA-C-C$_s$A, tRNA in which the last phosphodiester linkage is replaced by a phosphorothioate diester linkage; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

Deamination of [^{35}S]*C>pS*. The [^{35}S]Cp>S obtained from the DEAE-Sephadex column was deaminated by treatment with a mixture of 1.5 M NaH_2SO_3 (1:4, v/v) for 24 h at room temperature (Hayatsu et al., 1970). Desalting of this mixture by passage over Bio-Gel followed by DEAE-Sephadex was incomplete. The final separation from salt was achieved by preparative paper electrophoresis at pH 7.5 (0.1 M triethylammonium bicarbonate). All radioactive material recovered from this electrophoresis was chromatographed again on a DEAE-Sephadex column where it was separated into two compounds, UMPS (1.71×10^6 cpm) and U>pS (84 A_{260} units, 6.85×10^6 cpm). The total recovery after deamination was 3 mg (53%).

^{31}P NMR spectra of the mononucleotides were recorded in D_2O on a Bruker HFX 60 spectrometer equipped with a Fourier-transform unit (Bruker-Data System B-NC 12). The spectra of tRNA were measured on a Bruker WH 270 spectrometer in D_2O containing 50 mM Tris-HCl, pH 7.6, and 10 mM Mg^{2+}. The external standard was 85% aqueous H_3PO_4. Chemical shifts are expressed in δ values relative to this standard.

Results

Both diastereomers of ATPαS were tested as possible substrates for tRNA nucleotidyltransferase. ATPαS, isomer A, proved to be a substrate (K_m = 1.9 mM) with rates of incorporation similar to ATP (K_m = 0.6 mM), whereas ATPαS, isomer B, was not a substrate but a competitive inhibitor (K_i = 1.2 mM). tRNA-C-C$_{\bar{s}}$A could thus be prepared using ATPαS, isomer A, and tRNA-C-C as substrates under the same conditions and in as high a yield as tRNA-C-C-A. The limited digestion of this tRNA with RNase A allowed us to isolate C>pS after purification over Sephadex and DEAE-Sephadex. A ^{31}P NMR (Figure 3) spectrum of this material showed a single peak with a chemical shift of −74.58 ppm. This material was deaminated as described under Methods. The [^{35}S]U>pS recovered (3 mg) was considered to be too little for recording a reliable NMR spectrum or for crystallization. Therefore, it was mixed with authentic U>pS endo isomer (21 mg) and recrystallized from EtOH (2 mL). Crystalline [^{35}S]U>pS (17.6 mg, 73%) containing a total of 4.90×10^6 cpm (72%) was obtained (mp 198–205 °C).

Discussion

The formation of polynucleotides is catalyzed by such enzymes as template-dependent or template-independent DNA and RNA polymerases. tRNA nucleotidyltransferase can be considered as a degenerated RNA polymerase which can, under normal conditions, only polymerize a limited number of CTP and ATP molecules onto tRNA which lacks the CCA end as primer. Little is known about the mechanisms by which these enzymes bring about the formation of the internucleotidic bond. One of the few enzymes where the process of phosphodiester bond making or breaking is reasonably well understood is pancreatic RNase A. However, in this case, the enzyme takes a 2′,3′-cyclic phosphate and not a triphosphate as substrate (Richards and Wyckoff, 1971).

We are presently engaged in a study of the mechanism of polymerases involving identification of essential amino acid residues in *Escherichia coli* DNA-dependent RNA polymerase as well as an investigation of the stereochemistry of phosphodiester bond formation by this enzyme (Eckstein et al., 1976; Armstrong et al., 1976a, b). It is tempting to apply methods which have been successfully used with this RNA polymerase to tRNA nucleotidyltransferase where possible. Such comparative studies might reveal features common to both types of enzymes. In the present study, we have used ATPαS to investigate some stereochemical aspects of phosphodiester bond formation of tRNA nucleotidyltransferase from baker's yeast and to compare the results obtained with those from DNA-dependent RNA polymerase from *E. coli*.

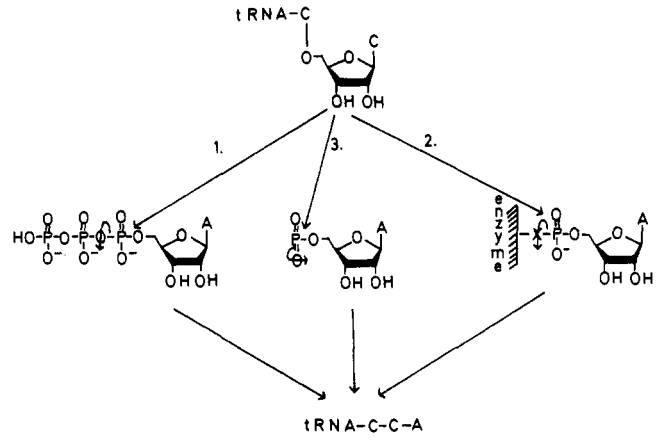

FIGURE 4: Mechanisms for enzymatic addition of ATP to tRNA-C-C.

The phosphodiester bond formation catalyzed by the transferase or any DNA or RNA polymerase can principally follow three different mechanisms (Figure 4). (1) The reaction could be an S_N2-type mechanism in which the nucleophile, the 3′-hydroxyl group of the tRNA, displaces pyrophosphate from CTP or ATP by nucleophilic attack at the α-phosphorus. This mechanism would result in inversion at this phosphorus if it were initially chiral. (2) Two consecutive S_N2-type reactions could take place, the first by attack of a nucleophilic group at the active site of the enzyme to form an intermediate which could in turn react with the 3′-hydroxyl group of the tRNA. Two consecutive inversions would result in retention of configuration at the α-phosphorus if it were initially chiral. It should be mentioned that no adenylated enzyme intermediate has so far been identified for this enzyme. (3) The reaction could proceed by elimination of pyrophosphate from CTP or ATP to form cytidine or adenosine metaphosphate which could then react in an addition reaction with the 3′-hydroxyl group of tRNA. This reaction may or may not result in racemization. The nucleophilic substitution reactions discussed under 1 and 2 could proceed via a pentacoordinated phosphorus intermediate, as discussed in more detail elsewhere (Eckstein et al., 1976).

We have already shown that ATPαS is a substrate for the transferase (Schlimme et al., 1970). Using tRNA^{Phe}-C-C it was possible to incorporate an AMPS residue. The tRNA^{Phe}-C-C$_{\bar{s}}$A in turn was a substrate for the corresponding aminoacyl synthetase. At that time, the experiments were carried out with a sample of chemically synthesized ATPαS which contained the two diastereomers in a ratio of approximately 1:1. Recently, we have described the preparation of ATPαS by a combination of chemical and enzymatic methods which yields the diastereomers, arbitrarily named A and B, in pure form (Eckstein and Goody, 1976). When [^{35}S]ATPαS, isomer A, is used as substrate for the transferase, one can isolate [^{35}S]tRNA^{Phe}-C-C$_{\bar{s}}$A in good yield. The ^{31}P NMR spectrum of this tRNA is worthy of note because the signal of the phosphorothioate has a chemical shift of −56.76 ppm and is thus far removed from all the other signals which cluster around +0.9 ppm (Gueron and Shulman, 1975). Because the

determination of configuration of the last internucleotidic linkage, the phosphorothioate diester bond, is not feasible at the polymer level at the present time, it has to be transformed into a compound whose configuration can be determined. The steps which lead to this compound must proceed by mechanisms whose stereochemical course is known. Degradation of [^{35}S]tRNA^{Phe}-C-C$_{\bar{S}}$A by pancreatic RNase A to [^{35}S]C>pS is the method of choice for this particular problem. The stereochemistry of transesterification of pyrimidine nucleoside 3′-phosphorothioate esters to pyrimidine nucleoside 2′,3′-cyclic phosphorothioates has been elucidated with the aid of the diastereomers of uridine 2′,3′-cyclic phosphorothioate (Usher et al., 1972). The enzyme follows an in-line mechanism. It has been shown that during hydrolysis by RNase A uridine 3′-phosphorothioate methyl ester is transesterified to the endo isomer of uridine 2′,3′-cyclic phosphorothioate (Saenger et al., 1974).

Hydrolysis of [^{35}S]tRNA^{Phe}-C-C$_{\bar{S}}$A by RNase A, in the presence of Mg^{2+} to suppress hydrolysis in other parts of the tRNA than the CCA end, produces in high yield [^{35}S]C>pS which is separated from the polymer by gel filtration. Analysis by ^{31}P NMR showed only one multiplet signal at −74.5 ppm. Chemically synthesized C>pS exhibits two signals (−74.3 and −75.6 ppm) which correspond to the chemical shifts observed with the two diastereomers of U>pS (−74.2 ppm (endo) and −75.6 ppm (exo)) (Eckstein et al., 1976). The chemical shift of the [^{35}S]C>pS would therefore indicate the presence of the endo isomer. To avoid any ambiguity in the assignment of the configuration, C>pS was deaminated by the method of Hayatsu et al. (1970). Some difficulty was encountered in desalting the sample. Purification by preparative paper electrophoresis and subsequent chromatography on DEAE-Sephadex was eventually successful. Approximately 3 mg of [^{35}S]U>pS was obtained and judged to be too little for an NMR spectrum as well as crystallization. Authentic crystalline endo isomer (21 mg) was therefore added for cocrystallization; 73% of crystalline material could be reisolated. Of the radioactivity, 72% was recovered in the crystals. This is good evidence that U>pS isolated from tRNA is the endo isomer, the more so because the exo isomer has never been obtained in crystalline form. A control experiment where authentic endo isomer of U>pS was subjected to the deamination treatment showed that the ^{31}P NMR spectrum remained unchanged. The deamination procedure, therefore, does not alter the configuration of the cyclic phosphorothioate group. It follows that C>pS also possessed the endo configuration. From the previous results with RNase A cited above, it is known that the endo isomer is the product of the transesterification of a phosphorothioate internucleotidic linkage with the *R* configuration (Saenger et al., 1974).

It is therefore concluded that ATPαS, isomer A, is condensed onto tRNA^{Phe}-C-C by tRNA nucleotidyltransferase from baker's yeast to produce a phosphorothioate linkage with the *R* configuration. Clearly, until the absolute configuration of ATPαS, isomer A, is established one is not able to draw conclusions as to whether this reaction has occurred with inversion or retention of configuration. The only statement which can be made is that no racemization has taken place. This, however, doesn't necessarily rule out mechanism 3 but restricts the reaction of adenosine metaphosphate—if it is at all on the pathway of this reaction—with the tRNA to an addition from one side and not from two.

A comparison with the results obtained recently with DNA-dependent RNA polymerase from *E. coli* (Eckstein et al., 1976) reveals that both enzymes have the same overall stereochemistry in the mechanism of phosphodiester bond formation. Both enzymes also only take the A isomer of ATPαS as substrate, whereas the B isomer is a competitive inhibitor. Preliminary experiments with tRNA nucleotidyltransferase from *E. coli* indicate that this enzyme, too, accepts only ATPαS, isomer A, as substrate and that isomer B is an inhibitor. Further investigations are required to determine whether this is a common feature for nucleoside 5′-triphosphate polymerizing enzymes. Such experiments are in progress.

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