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ARTICLE *in* BIOCHEMISTRY · JUNE 1982

Impact Factor: 3.02 · DOI: 10.1021/bi00539a042 · Source: PubMed

Stereochemical Course of Nucleotidyl Transfer Catalyzed by Bacteriophage T7 Induced DNA Polymerase†

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ABSTRACT: The bacteriophage T7 induced DNA polymerase, consisting of the phage specified gene 5 protein associated with *Escherichia coli* thioredoxin, catalyzes the copolymerization of S_P-dATPαS with dTTP, producing the alternating copolymer poly[d(T_sA)] by a mechanism involving inversion of configuration at P_α. Degradation of poly[d(T_sA)] by the nucleolytic action of *E. coli* DNA polymerase I produced the dinucleotide pdTp$_s$dA, whose configuration at the phosphorothioate diester was assigned as *R* by comparison of the phosphorus-31 nuclear magnetic resonance chemical shift (55.0 ppm downfield from H_3PO_4) with that of an authentic sample. Further degradation by alkaline phosphatase to R_P-dTp$_s$dA (55.6 ppm downfield from H_3PO_4) confirmed the configuration. The stereochemistry provides no evidence of a double displacement mechanism.

The stereochemical course of nucleotidyl transfer catalyzed by DNA polymerase I from *Escherichia coli* has recently been shown to proceed with inversion of configuration at P_α of the S_P epimer of dATPαS[1] (Burgers & Eckstein, 1979; Brody & Frey, 1981). Accumulated evidence has established the general pattern that enzymatic substitutions at phosphorus proceed with inversion of configuration when the reaction occurs via a single displacement mechanism, while double displacements proceed with overall retention (Sheu et al., 1979; Blättler & Knowles, 1979). On this basis it was concluded that DNA polymerase I most probably catalyzes polymerization by a single displacement mechanism.

The bacteriophage T7 induced DNA polymerase differs from *E. coli* DNA polymerase I and other DNA polymerases in that it is a complex of two proteins associated in equimolar amounts (Modrich & Richardson, 1975; Hori et al., 1979; Adler & Modrich, 1979). One protein (M_r 80000) is encoded by the viral gene 5 and the other (M_r 12000) is the host protein thioredoxin. The gene 5 protein lacks the polymerase and double-stranded exonuclease activities of the complex but retains 3′–5′ exonucleolytic activity toward single-stranded DNA. Purified thioredoxin from *E. coli* forms a molecular complex with the gene 5 protein, which exhibits polymerase and nucleolytic activities similar to those of the purified complex.

While it is clear that thioredoxin plays an essential role in supporting the polymerase activity of phage T7 induced DNA polymerase, the molecular basis for its involvement is not known. The report that *E. coli* thioredoxin is fully reduced with one of the two cysteinyl sulfhydryl groups in its active site phosphorylated in vivo (Pigiet & Conley, 1978; Conley & Pigiet, 1978) suggests the possibility that thioredoxin might be directly involved in the mechanism of action of the T7 DNA polymerase. One possible direct role would be for either the phosphoryl or the sulfhydryl group of phosphothioredoxin to mediate nucleotidyl transfer between deoxynucleoside 5′-triphosphates and the 3′-OH end of the growing chain by a double displacement mechanism involving a deoxyadenylyl–phosphothioredoxin intermediate. In this paper we show that the enzyme catalyzes polymerization of S_P-dATPαS with inversion of configuration at P_α, consistent with a mechanism involving a single displacement at P_α.

Materials and Methods

S_P-dATPαS,$\alpha^{18}O_2$ was synthesized by stereospecific phosphorylation of dAMPS,$^{18}O_2$ with phosphoenolpyruvate and a catalytic amount of ATP catalyzed by the coupled actions of adenylate kinase and pyruvate kinase as described by Brody & Frey (1981). dAMPS,$^{18}O_2$ was synthesized as described by Brody & Frey (1981). Primer d(A-T) was obtained from P-L Biochemicals, DNase I from Worthington, and alkaline phosphatase from Sigma.

Nonenzymatically synthesized ($R_P + S_P$)-dTp$_s$dA was synthesized by a method analogous to that of Lesnikowski et al. (1978). Bacteriophage T7 induced DNA polymerase and DNA polymerase I were purified as described by Adler & Modrich (1979) and Jovin et al. (1969), respectively. Thin-layer chromatography of nucleotides was carried out by using Eastman silica gel plates containing a fluorescent indicator with 1-propanol/concentrated ammonia/water, 6:3:1, as the mobile phase.

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§ Supported by National Institutes of Health Training Grant GM 07171.

[1] Abbreviations: dAMPS,^{18}O, 2′-deoxyadenosine 5′-*O*-[^{18}O]-phosphorothioate; dATPαS, 2′-deoxyadenosine 5′-*O*-(1-thiotriphosphate); dATPαS,$\alpha^{18}O$, 2′-deoxyadenosine 5′-*O*-(1-thio[1-^{18}O]triphosphate); pdTp$_s$dA, 3′-*O*-(5′-phospho-2′-deoxythymidyl)-5′-*O*-(2′-deoxyadenosyl) phosphorothioate; dTp$_s$dA, 3′-*O*-(2′-deoxythymidyl)-5′-*O*-(2′-deoxyadenosyl) phosphorothioate; poly[d(T_sA)], alternating copolymer of 2′-deoxyadenosine 5′-*O*-phosphorothioate and 2′-deoxythymidine 5′-phosphate; R_P, the *R* configuration of a phosphorus in a nucleotide; S_P, the *S* configuration of a phosphorus in a nucleotide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; A_{260} units, absorbance at 260 nm if the entire sample were in 1 mL.

0006-2960/82/0421-2570$01.25/0

Nuclear magnetic resonance spectra were obtained with a Bruker WP-200 spectrometer. The proton spin decoupled ^{31}P nuclear magnetic resonance spectra were obtained with the spectrometer field frequency locked at the deuterium resonance in 33% D_2O. All chemical shifts were referenced to that of 85% H_3PO_4 as an external standard.

Results and Discussion

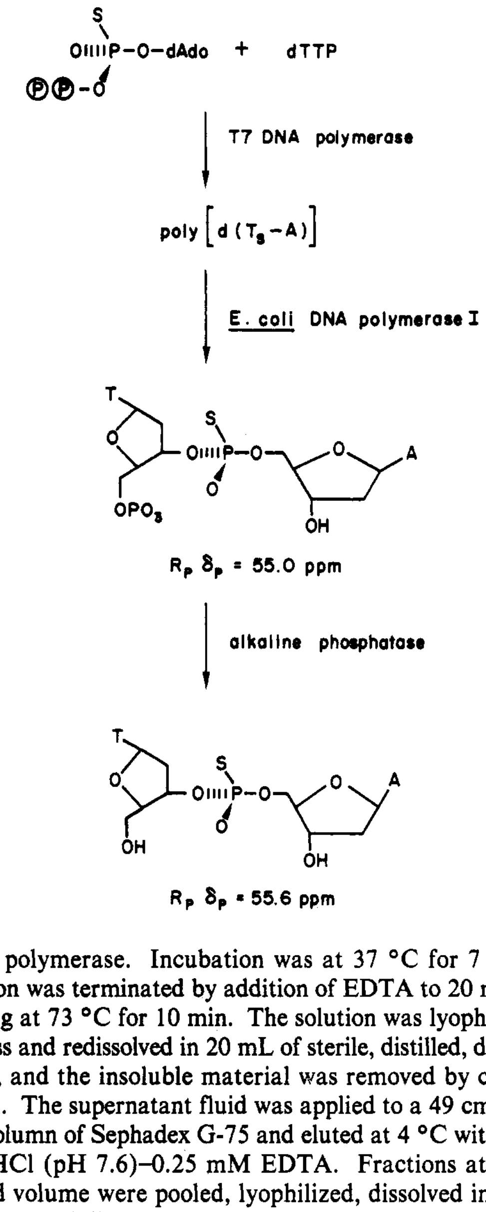
Configurational Analysis. In the earlier study of DNA polymerase I from *E. coli*, S_P-dATPαS,$\alpha^{18}O_2$ was synthesized and polymerized with dTTP in the presence of poly[d(A-T)] as the primer template to poly[d(T_sA)], the alternating copolymer of 2′-deoxyadenosine 5′-*O*-[^{18}O]phosphorothioate and 2′-deoxythymidine 5′-phosphate. Poly[d(T_sA)] was degraded by the exonucleolytic action of DNA polymerase I to the dinucleotide [^{18}O]pdTp_sdA enriched with ^{18}O at the phosphorothioate diester linkage; and this dinucleotide was characterized with respect to its proton and phosphorus nuclear magnetic resonance spectra. The dinucleotide was further degraded by treatment with anhydrous hydrazine to remove the thymine ring, followed by treatment of the resulting hydrazone with base to eliminate dAMPS,^{18}O. The configuration at phosphorus of this sample of dAMPS,^{18}O was shown to be *R*, corresponding to inversion of P_α in the polymerization of S_P-dATPαS,$\alpha^{18}O_2$.

In addition to unmasking the stereochemical course of the DNA polymerase reaction, the earlier study correlates the phosphorus configuration of R_P-[^{18}O]pdTp_sdA with those of the substrate S_P-dATPαS,$\alpha^{18}O_2$ and the ultimate degradation product R_P-dAMPS,^{18}O. Since the phosphorus NMR chemical shift of pdT$_s$dA is expected to differ for the two epimers with different configurations at the chiral phosphorus, by analogy with the epimers of ATPαS and ADPαS (Sheu & Frey, 1977; Jaffe & Cohn, 1978) and other phosphorothioate diesters (Niewiarowski et al., 1980), it should now be possible to assign the configuration of an unknown sample by means of this shift, which is known for the R_P epimer. Thus the general procedure for establishing the stereochemical course of DNA polymerases, which was developed and applied to *E. coli* DNA polymerase I, can be shortened significantly because the configuration of pdTp_sdA should be assignable by ^{31}P nuclear magnetic resonance; and ^{18}O should no longer be needed because [^{18}O]pdTp_sdA would not be further degraded to dAMPS,^{18}O for configurational analysis.

In this study of T7-induced DNA polymerase the shorter procedure has been adopted. Isotopically enriched S_P-dATPαS,$\alpha^{18}O_2$ was used as the substrate so that in case of unanticipated complications with the assignment of configuration to [^{18}O]pdTp_sdA the dinucleotide could be degraded to dAMPS,^{18}O for configurational analysis. The ^{18}O-induced shift of about 0.02 ppm in the ^{31}P nuclear magnetic resonance signal (Cohn & Hu, 1978) would not interfere because the ^{31}P chemical shifts of the two possible epimers were expected to differ by 0.2–0.4 ppm.

Stereochemical Course of Polymerization by Phage T7 DNA Polymerase. The stereochemical analysis is outlined in Scheme I. S_P-dATPαS,$\alpha^{18}O_2$ and dTTP were polymerized by the action of T7 phage induced DNA polymerase to poly[d(T_sA)] in a 200-mL reaction containing 50 mM Tris-HCl (pH 7.6), 6.25 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 3.5 μM d(A-T) primer, 1 mM dTTP, 0.5 mM S_P-dATPαS,$\alpha^{18}O_2$, 4.3 ng/mL DNase I, and 0.3 μg/mL T7 DNA polymerase. Incubation was at 37 °C for 7 h. The reaction was terminated by addition of EDTA to 20 mM and heating at 73 °C for 10 min. The solution was lyophilized to dryness and redissolved in 20 mL of sterile, distilled, deionized water, and the insoluble material was removed by centrifugation. The supernatant fluid was applied to a 49 cm × 12.5 cm^2 column of Sephadex G-75 and eluted at 4 °C with 5 mM Tris-HCl (pH 7.6)–0.25 mM EDTA. Fractions at the excluded volume were pooled, lyophilized, dissolved in 12 mL of water, and dialyzed vs. 20 mM potassium phosphate (pH 7.4)–0.1 mM EDTA. Analysis on 1% agarose gels run under denaturing conditions (0.1 N NaOH) indicated a polymer length of about 1500 nucleotides.

Scheme I



Digestion of poly[d(T_sA)] to pdTp_sdA by *E. coli* DNA polymerase I was in a 50-mL reaction containing 170 A_{260} units of polymer, 50 mM potassium phosphate (pH 7.4), 7.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 12.5 μg/mL bovine serum albumin, and 6.4 μg of DNA polymerase I. Incubation was at 37 °C for 5 h, and then the reaction was terminated by cooling to 0 °C and then addition of EDTA to 10 mM.

After the *E. coli* DNA polymerase I catalyzed degradation of poly[d(T_sA)] to pdTp_sdA, the dinucleotide was purified from the reaction mixture by chromatography through a 2.3 × 42 cm column of DEAE-Sephadex A-25 in the bicarbonate form. The column was eluted at 4 °C with a linear gradient consisting of 1.5 L of 0.2 M and 1.5 L of 0.45 M triethylammonium bicarbonate buffers, both at pH 7.5. The major nucleotide containing band appeared at about 0.35 M triethylammonium bicarbonate. The appropriate fractions were pooled and evaporated to dryness by in vacuo rotary flash evaporation, bath temperature <35 °C. The residue was dissolved in ethanol and again evaporated to dryness. The recovery of pdTp$_s$dA was 280 A_{260} units, corresponding to 11.3 μmol. This material was identical by the criteria of thin-layer chromatography and proton spin decoupled ^{31}P nuclear magnetic resonance to the sample of pdTp$_s$dA obtained in the earlier study of *E. coli* DNA polymerase I (Brody & Frey, 1981). The ^{31}P nuclear magnetic resonance spectrum consisted of a singlet at 3.9 ppm upfield from H_3PO_4 (1 P) assigned to the 5′-phosphate and a singlet at 55.0 ppm downfield from H_3PO_4 (1 P) assigned to the 3′,5′-phosphorothioate bridge of pdTp$_s$dA.

Since the chemical shift of the phosphorothioate diester bridge was indistinguishable from that of our earlier sample of R_P-pdTp$_s$dA, the configuration R was tentatively assigned. This assignment could not be definitive because the S_P epimer was not available for comparison. However, the dephosphorylated dinucleotide R_P-dTp$_s$dA was available from the earlier study (Brody & Frey, 1981), and we were supplied with a synthetic sample of (R_P + S_P)-dTp$_s$dA for comparison. The pdTp$_s$dA was, therefore, degraded to dTp$_s$dA by the action of alkaline phosphatase in a reaction mixture consisting of 6 μmol of pdTp$_s$dA and 3 units of alkaline phosphatase in 3 mL of 0.1 M triethylammonium bicarbonate buffer at pH 8.0. After 3 h no pdTp$_s$dA remained. The proton-decoupled ^{31}P nuclear magnetic resonance spectrum of this reaction mixture consisted of two singlet signals, one at 55.6 ppm downfield from H_3PO_4 assigned to dTp$_s$dA and one at 2.8 ppm downfield from H_3PO_4 assigned to inorganic phosphate. Identical chemical shifts were measured after similar degradation of pdTp$_s$dA obtained in the earlier study (Brody & Frey, 1981). These were compared with the chemical shifts of 55.6 and 55.0 ppm downfield from H_3PO_4 measured for the synthetic sample of dTp$_s$dA. This comparison confirmed the configurational assignment of R_P for the epimer exhibiting the ^{31}P shift of 55.6 ppm downfield from H_3PO_4.

We conclude that polymerization of S_P-dATPαS catalyzed by T7 phage induced DNA polymerase proceeds with inversion of configuration at P_α as does the polymerization by *E. coli* DNA polymerase I. The stereochemistry is consistent with and suggests a single displacement mechanism for both polymerases; in particular, the stereochemistry does not indicate the involvement of either a phosphorothioate or sulfhydryl group of phosphothioredoxin as nucleophilic catalysts mediating adenylyl group transfer by a double displacement mechanism.

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