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Mechanistic Studies on Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from *Escherichia coli* Using Phosphorothioate Analogues. 2. The Elongation Reaction†

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ABSTRACT: The metal ions Mn(II), Co(II), Zn(II), and Cd(II) are able to replace Mg(II) in the transcription of poly[d(A-T)] by deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase from *Escherichia coli*. In the presence of Mg(II), the A isomers of adenosine 5′-*O*-(1-thiotriphosphate) (ATPαS) and uridine 5′-*O*-(1-thiotriphosphate) (UTPαS) are substrates for RNA polymerase (K_m = 0.2–0.4 mM) whereas the B isomers are weak inhibitors ($K_i \simeq 2$ mM). Replacement of Mg(II) by Co(II) or Cd(II) does not alter the substrate properties of these analogues, the A isomers remaining substrates while the B isomers are inactive. Both diastereomers of adenosine 5′-*O*-(2-thiotriphosphate) (ATPβS) are substrates with Mg(II), having similar K_m values [0.3–0.4 mM, K_m(MgATP) = 0.05 mM]. However, with Cd(II) only the B isomer is a substrate [K_m = 0.8 mM, K_m(CdATP) = 1.2 mM], the A isomer being a weak inhibitor ($K_i \geq 5$ mM). Since Cd(II) chelates mainly to the sulfur atom of phosphorothioates and Mg(II) to the oxygen atom [Jaffe, E. K., & Cohn, M. (1978) *J. Biol. Chem. 253*, 4823–4825], a model is proposed on the basis of these data for the binding of the metal–ATP complex to the elongation site of RNA polymerase. Thus, the Δ isomer of the bidentate MgATP complex [Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3279–3286] is the active form for RNA polymerase, and there is an additional binding of the nonchelated β-oxygen to the enzyme. The metal atom is not bound to the α-phosphate, but there is a stereospecific interaction between a positive group on the enzyme and one of the α-phosphate atoms.

The syntheses and separation of the diastereomers of ATPαS[1] and ATPβS (Eckstein & Goody, 1976) have enabled their use in the study of enzyme mechanisms (Eckstein, 1975, 1979). The asymmetry resulting from the introduction of the phosphorothioate group can be utilized to investigate not only the stereochemistry of phosphodiester bond formation [for reviews, see Eckstein (1979) and Westheimer (1979)] but also the geometry of the substrate binding site by comparing the substrate properties of the two diastereomers. Furthermore, information as to the active form of the divalent metal ion–ATP complex can be derived by the use of metal ions differing in their affinities for the oxygen and sulfur atoms of the phosphorothioate groups. Thus, on the basis of nuclear magnetic resonance studies, Jaffe & Cohn (1978) concluded that Mg(II) chelates preferentially to the oxygen atom of the β-phosphorus of ATPβS but that Cd(II) chelates to the sulfur atom. In the presence of Mg(II), the B isomer of ATPβS is the preferred substrate for hexokinase, the A isomer being inactive, whereas with Cd(II) the reverse is true, the A isomer being a substrate and the B isomer inactive. In the case of Co(II), both isomers are substrates.

The stereochemistry of phosphodiester bond formation has been determined for the elongation reaction (Eckstein et al., 1976; Burgers & Eckstein, 1978) and for the initiation and pyrophosphate exchange reactions (Yee et al., 1979) of DNA-dependent RNA polymerase from *Escherichia coli*. This enzyme requires a divalent metal ion such as Mg(II) for its polymerizing activity, and nuclear magnetic resonance studies with Mn(II) in place of Mg(II) (Koren & Mildvan, 1977) have indicated that there is one tight binding site for this ion on the enzyme. In this investigation we report the stereospecificity of incorporation of phosphorothioate substrate

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[1] The abbreviations used are as in the previous paper (Yee et al., 1979).

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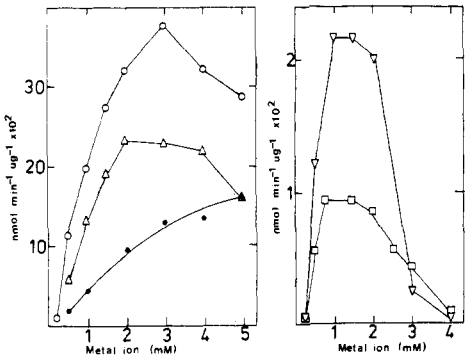

FIGURE 1: Effect of divalent metal ions on the transcription of poly[d(A-T)] by RNA polymerase. The assay solution was as described under Experimental Section and contained 5.8 μg of enzyme, 1 mM [^{14}C]ATP, 1 mM UTP, and the following divalent metal ions: Mg(II) (●); Mn(II) (○); Co(II) (△); Zn(II) (▽); Cd(II) (□).

analogues with RNA polymerase in the presence of different divalent metal ions and on the basis of these data suggest a model for the binding of the substrate triphosphate to the elongation site.

Experimental Section

Materials. Unlabeled nucleotides were products of Pharma-Waldhof, and labeled nucleotides were obtained from Amersham Buchler. Poly[d(A-T)] was a product of Miles Laboratories. The diastereomers of ATPαS, ATPβS, and UTPαS were prepared as described in the preceding paper (Yee et al., 1979).

Enzyme. RNA polymerase holoenzyme was purified by a published procedure (Sternbach et al., 1975) and was generously donated by Dr. H. Sternbach. Since the RNA polymerase storage buffer normally contains magnesium, this was removed in order to investigate the effect of different divalent metal ions on enzyme activity. The enzyme was dialysed against (1) 40 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol for 4.5 h with one buffer change, (2) the same buffer containing 10% glycerol (v/v) for 2.5 h with one buffer change, and (3) 10 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 50% (v/v) glycerol for 14 h. The enzyme was stored for the duration of the studies in the latter buffer at −20 °C, and no detectable loss in activity was observed after 2 months.

Methods. Enzyme Assay. The enzyme was assayed in a solution (0.1 mL) containing 40 mM Tris-HCl, pH 8.0, 200 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1.0 A_{260} unit/mL poly[d(A-T)], divalent metal ion in the form of its chloride salt, and nucleotides as described in the legends to the figures and tables. Incubation was at 37 °C, and 30–50-μL aliquots were removed after various times and streaked onto a Whatmann 3MM paper strip (2 × 13.5 cm) which had been pretreated with 50 μL of 0.3 M EDTA. The strip was then developed in 1 M ammonium acetate–ethanol (1:1 v/v) by descending chromatography. After the strip was dried, the origin (2 × 2 cm) was cut out and the radioactivity measured in 6 mL of a toluene-based scintillation fluid.

Results

Activity of RNA Polymerase in the Presence of Various Divalent Metal Ions. The divalent metal ions Mn(II), Co(II), Zn(II), and Cd(II) were all able to support transcription of poly[d(A-T)] by RNA polymerase (Figure 1). However, they showed optimum activity at concentrations lower than that observed with Mg(II), and at higher concentrations they were inhibitory. No activity was observed in the presence of Ca(II), Ni(II), or Cu(II).

Table I: Transcription of ATPαS on Poly[d(A-T)] in the Presence of Different Metal Ions[a]

	nmol of ^{14}C-labeled nucleotides incorporated/min			
	UTPαS isomer		ATPαS isomer	
metal ion	A	B	A	B
10 mM Mg(II)[b]	0.62	0.03	0.18	0.01
2 mM Co(II)[b]	0.75	0.04	0.32	0.01
2 mM Cd(II)[c]	0.15	0.007	0.0014	0.0004

[a] The assay solution was as described under Experimental Section and contained 1 mM phosphorothioate analogue and in the case of UTPαS, 1 mM [^{14}C]ATP, and for ATPαS, 1 mM [^{14}C]UTP. The metal ion concentration was as indicated, and the amount of enzyme is indicated in footnotes *b* and *c*. [b] 5.8 μg. [c] 28 μg.

Table II: Initial Rates for the Transcription of Poly[d(A-T)] with ATPβS, Isomers A and B, in the Presence of Different Divalent Metal Ions[a]

metal ion	ATPβS, isomer A	ATPβS, isomer B	B/A
10 mM Mg(II)	34.5	29.8	0.9
2 mM Mn(II)	55.2	92.4	1.7
2 mM Co(II)	36.0	74.8	2.1
1 mM Zn(II)	1.6	8.4	5.3
2 mM Cd(II)	≤0.03	9.0	≥300

[a] The assay solution was as described under Experimental Section and contained the metal ion as indicated, 1 mM ATPβS, isomer A or B, 1 mM [^{14}C]UTP, and 5.8–28 μg of enzyme. The solution was incubated at 37 °C, and aliquots were removed after various times in order to determine the initial velocity. Rates are expressed as nmol min^{-1} mg^{-1}.

Substrate Properties of UTPαS and ATPαS, Isomers A and B, in the Presence of Various Divalent Metal Ions. We have previously shown (Eckstein et al., 1976) that the A isomer of ATPαS is a substrate for RNA polymerase in the presence of Mg(II), whereas the B isomer is only a weak competitive inhibitor. Similar data were also obtained for UTPαS with Mg(II), the A isomer being a substrate with K_m = 0.25 mM and V_{max} = 0.29 μmol min^{-1} mg^{-1} [K_m(UTP) = 0.06 mM, V_{max} = 0.22 μmol min^{-1} mg^{-1}] and the B isomer being a weak inhibitor with K_i = 1.8 mM. However, the latter inhibition was not purely conpetitive and proved to be of the mixed type.

Replacement of Mg(II) by Co(II) or Cd(II) did not affect the substrate properties of the disastereomers of either UTPαS or ATPαS (Table I). Thus, the A isomers were able to support the transciption of poly[d(A-T)], but the B isomers showed only a low incorporation of labeled nucleotide. The small amount of RNA produced in the latter case may have been due to traces of the A isomer or desulfurized material (i.e., ATP or UTP) rather than genuine incorporation of the B isomer. This is supported by the observation that after an initial "burst" of incorporation the amount of label incorporated then remained constant over the incubation period (results not shown). Similar results (not shown) were observed with Mn(II) and Zn(II).

Substrate Properties of ATPβS, Isomers A and B, in the Presence of Various Divalent Metal Ions. Both isomers of ATPβS were almost equally effective as substrates for RNA polymerase in the prescence of Mg(II) (Table II). However, with the other metal ions an increasing preference for the B isomer was observed in the order Mn(II) < Co(II) < Zn(II) ≪ Cd(II). This sequence would also be that expected for the preferred chelation to sulfur over oxygen (Pearson, 1966). By use of Cd(II), the enzyme proved to be almost entirely ste-

Table III: Steady-State Kinetic Constants for ATPβS, Isomers A and B, with either Mg(II) or Cd(II) as Divalent Metal Ion Activator[a]

substrate	K_m (mM)	K_i (mM)	V_{max} (μmol min^{-1} mg^{-1})
MgATP	0.05 ± 0.02		0.16 ± 0.02
MgATPβS (A)	0.38 ± 0.05		0.07 ± 0.02
MgATPβS (B)	0.33 ± 0.05		0.06 ± 0.02
CdATP	1.3 ± 0.2		0.07 ± 0.02
CdATPβS (A)		≥5[b]	
CdATPβS (B)	0.8 ± 0.2	0.6 ± 0.2[b]	0.04 ± 0.02

[a] General assay conditions were as described under Experimental Section. The enzyme (2.8 or 11.2 μg) was preincubated in the assay solution for 5 min at 37 °C together with the metal ion (10 mM $MgCl_2$ or 1 mM $CdCl_2$) and varying concentrations of the triphosphate whose K_m was to be determined. The polymerization was then started by addition of [^{14}C]UTP (14 000 cpm/nmol) to a final concentration of 1 mM. After a further 5 min at 37 °C, a 50-μL aliquot was removed to determine the initial velocity. Lineweaver–Burke plots were calculated by least-squares analysis. [b] The preincubation solution contained 1 mM ATPβS, isomer A or B, 0.2–1.0 mM [^{14}C]ATP, and 1 mM $CdCl_2$. Reaction was started by addition of UTP to a final concentration of 1 mM.

reospecific for the B isomer. Only a very low rate of incorporation was observed with the A isomer, which may have been caused by contamination with the B isomer or ATP.

Kinetic Constants for ATPβS, Isomers A and B, as Measured with Mg(II) or Cd(II). In order to investigate further the differences observed in the substrate properties of the diastereomers of ATPβS as measured with Mg(II) or Cd(II),[2] we determined their steady-state kinetic constants (Table III). The values obtained are apparent values since the data were derived by using the complete enzymatic reaction and not the ternary complex (Rhodes & Chamberlin, 1974). By use of Mg(II), the K_m values for both isomers of ATPβS were almost identical and about sevenfold greater than that for ATP. The V_{max} values were also similar and about half that for ATP. With Cd(II) the K_m for ATP was raised more than 20-fold compared to MgATP, whereas the V_{max} was only reduced by a third, indicating that the low activity observed with this metal ion (Figure 1) is primarily due to the high K_m for CdATP (and presumably UTP) and the nonsaturating concentrations employed. In contrast to Mg(II), the K_m for CdATPβS, isomer B, was similar to that for CdATP. The A isomer of ATPβS proved to be a very weak inhibitor of CdATP with an estimated K_i value of ≥5 mM. A more accurate determination of this constant, as well as the type of inhibition involved, was not possible since an increase in the nucleotide concentration depleted the system of free metal ion by complexation of the Cd(II) with the phosphorothioate. However, when ATPβS, isomer B, was employed as an inhibitor of CdATP at the same concentration as that used for the A isomer, a K_i of 0.6 mM was observed, the inhibition being clearly competitive. It is, therefore, apparent that CdATPβS, isomer B, binds to RNA polymerase at least 9 times more strongly that the A isomer.

Discussion

The divalent metal ions Mn(II), Co(II), Zn(II), and Cd(II) are able to replace Mg(II) in the transcription of poly[d(A-T)] by RNA polymerase. Previous reports have shown that Mn(II) can satisfy the divalent metal ion requirements of RNA polymerase (Furth et al., 1962; Chamberlin & Berg, 1962) and in the case of the *M. luteus* enzyme (Fox & Weiss, 1964) Co(II) as well. However, no activity was observed with the *E. coli* enzyme in the presence of either Co(II), Cd(II), or Zn(II). The reason for this discrepancy in the case of Zn(II) and Cd(II) is probably their low activity and inhibitory properties at higher concentrations. It must also be noted that the K_m values for the substrates in the presence of other metal ions may be much higher than that observed with Mg(II), as we have found with Cd(II), necessitating higher substrate concentrations in the enzyme assay.

Ribonucleoside 5′-(1-thiotriphosphates) are incorporated stereospecifically into RNA by RNA polymerase, the A isomers of ATPαS and UTPαS being the preferred substrates for this enzyme. Such stereospecificity has been observed with *E. coli* DNA polymerase I (Burgers & Eckstein, 1979a), *M. luteus* polynucleotide phosphorylase (Burgers & Eckstein, 1979b), yeast tRNA nucleotidyl transferase (Eckstein et al., 1977), and yeast phenylalanyl tRNA synthetase (von der Haar et al., 1977), all enzymes involving reaction at the α-phosphate and all taking the A isomer of the corresponding phosphorothioate stereospecifically. In the case of RNA polymerase, this stereospecificity was not affected by substitution of Mg(II) with either Mn(II), Co(II), Zn(II), or Cd(II), suggesting that chelation of the metal ion to the α-phosphate is not necessary for enzyme activity. Furthermore, the lack of substrate activity for the B isomers of the α-phosphorothioates is partly reflected in their weaker binding to the enzyme, the K_i values for these analogues being approximately 10-fold greater than the K_m values for the corresponding A isomers. Similar data have been observed with DNA polymerase I (Burgers & Eckstein, 1979a), and an analogous explanation can be proposed in which one of the oxygens on the α-phosphate binds to a positively charged group on the enzyme (see Figure 3). Due to the chirality of the α-phosphorothioates, only one of the isomers, in this case the A isomer, can satisfy this requirement.

In describing the potential role in enzymic reactions of bidentate complexes of MgATP in which the metal ion is chelated to the β- and γ-phosphates, Cornelius & Cleland (1978) have suggested the nomenclature Λ and Δ for the two possible isomers of these complexes (parts a and b of Figure 2). Using the stable bidentate complex $Co(NH_3)_4ATP$, they concluded that the Λ isomer is the active form for yeast hexokinase. In the case of phosphoribosyl pyrophosphate synthetase, the Δ isomer has been shown to be the active form (Li et al., 1978). For each of the diastereomers of ATPβS, two forms of the bidentate metal complex can be envisaged, depending upon whether the metal ion ligands through the sulfur or oxygen on the β-phosphorothioate (parts c–f of Figure 2), and these may be classified as Λ or Δ as judged by the geometry of the phosphate ligands to the metal ion.

In contrast to the α-phosphorothioates, both isomers of ATPβS proved to be substrates for RNA polymerase in the presence of Mg(II), but with Cd(II) only the B isomer was active. Since Cd(II) chelates to ATPβS primarily through the sulfur (Jaffe & Cohn, 1978), the bidentate forms of CdATPβS, isomers A and B, will be as represented in parts c and f of Figure 2, respectively, with the latter being the active form for RNA polymerase and corresponding to the Δ form of MgATP (Figure 2b). Mg(II), on the other hand, chelates primarily through oxygen, and the bidentate forms of the A and B isomers would be as in parts d and e of Figure 2, respectively. There is, therefore, at first sight a discrepancy since both isomers are equally effective as substrates with Mg(II), and yet the Cd(II) data indicate the Δ form of the MgATP complex to be the active form for RNA polymerase. A possible explanation for this phenomenon would invoke a

[2] Under the conditions employed for the kinetic measurements the isomers of ATPβS were hydrolyzed in the presence of Cd(II) to ADPβS with a half-life of 5 h.

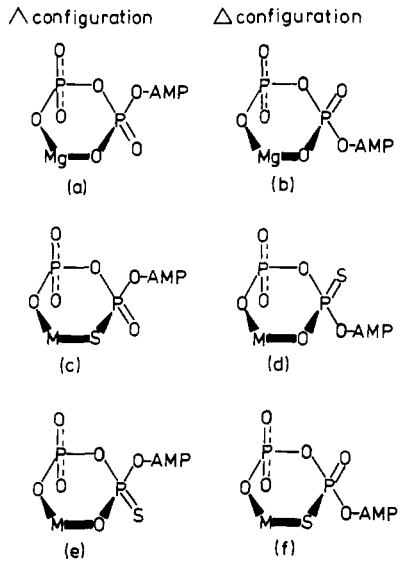

FIGURE 2: Absolute configurations of the bidentate metal ion complexes of ATP (a, b) and ATPβS, isomers A (c, d) and B (e, f).

group on the enzyme which interacts with the nonchelated β-oxygen with an affinity similar to that of Mg(II), e.g., through a hydrogen bond. Such a hypothesis has been suggested by W. W. Cleland (personal communication) to explain the nonstereospecificity of certain kinases. Two criteria would therefore be necessary for the binding of the β-phosphate. In the case of the β-phosphorothioates and Mg(II), these criteria are only partially fulfilled, resulting in less effective substrate activity compared to ATP. Thus, ATPβS, isomer A, is able to bind as the Δ complex with the preferred chelation of Mg(II) to oxygen but with an unfavorable hydrogen bond to the sulfur. In the case of the B isomer, the unfavorable Mg(II)–sulfur coordination necessary for the active form of the bidentate complex (Figure 2f) is offset by the favored hydrogen bond to the nonchelated oxygen. Both diastereomers of MgATPβS are therefore substrates for RNA polymerase (assuming that the loss of one binding criterion for the β-phosphate is not critical for enzyme activity). If one now considers the Cd(II) data, it can be seen that due to the preferred chelation of Cd(II) to sulfur the Δ form of isomer B (Figure 2) satisfies both criteria, whereas the Δ form of isomer A (Figure 2d) satisfies neither. Furthermore, the increasing stereospecificity for isomer B with the other divalent metal ions can then be explained by an increasing preference of the metal ion for sulfur over oxygen and the resulting Δ form of the B isomer in which both binding criteria are satisfied as compared to the Δ form of isomer A.

On the basis of these data, and knowing the absolute configurations of the diastereomers of ATPαS (Burgers & Eckstein, 1978) and ATPβS (Jaffe & Cohn, 1978) as well as the stereochemistry of phosphodiester bond formation (Burgers & Eckstein, 1978), we propose the stereochemical model shown in Figure 3 as representing the binding of the triphosphate moiety of the nucleoside 5′-triphosphate to the elongation site of RNA polymerase. This may be summarized as follows. (1) MgATP is bound to RNA polymerase as a β,γ-bidentate complex in the Δ configuration. (2) The oxygen atom of the β-phosphate not chelated to the metal atom interacts with the enzyme, possibly through a hydrogen bond. (3) The charge on the α-phosphate is neutralized by a specific interaction between a positive group on the enzyme and one of the α-phosphate oxygen atoms. (4) Attack of the terminal 3′-OH group of the growing RNA chain (or initiator) and subsequent release of pyrophosphate occurs by an in-line geometry. The model presented here is virtually identical with

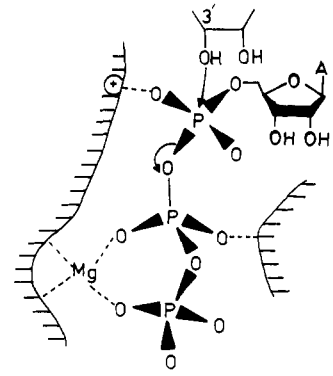

FIGURE 3: Model of the absolute configuration of MgATP at the elongation site of RNA polymerase.

that for DNA polymerase (Burgers & Eckstein, 1979a) with the exception of the β-phosphate oxygen interaction with the enzyme, which was observed with RNA polymerase but not with DNA polymerase.

Acknowledgments

The authors are indebted to Drs. P. M. J. Burgers and H. Wiedner for valuable advice and discussions and to G. Witzel for her excellent technical assistance.

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