Studies on the Mechanism of Ribonucleic Acid Synthesis. II. Stabilization of the Deoxyribonucleic Acid–Ribonucleic Acid Polymerase Complex by the Formation of a Single Phosphodiester Bond*

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ABSTRACT: The effects of the formation of a single phosphodiester bond on both the stability of the DNA–enzyme complex to dissociation by high ionic strength and on the sensitivity of the enzyme to the action of the antibiotic Rifampicin have been studied using the d(A-T) copolymer as template for the RNA polymerase of *Escherichia coli*. The specificity of the pyrophosphate exchange reaction with this enzyme and template has also been investigated. The formation of a single phosphodiester bond between ApU and ATP and between UpA and UTP has been shown to result in the stabilization of the DNA–enzyme complex to dissociation by high salt concentrations. Neither purine ribonucleoside triphosphates alone nor preformed oligonucleotides were able to stabilize the template–enzyme complex. Rifampicin was shown to inhibit phosphodiester bond formation between ApU and ATP and between UpA and UTP. Although preincubation of the DNA–enzyme complex with either ATP, UTP, ApU, or UpA did not protect the enzyme against the inhibitory action of Rifampicin, the formation of a phosphodiester between ApU and ATP (ApUpA) rendered the enzyme resistant to Rifampicin action.

The analogous formation of the trinucleotide UpApU failed to protect the enzyme from the action of this antibiotic. With the d(A-T) copolymer as a template, the enzyme catalyzed a pyrophosphate exchange reaction with UTP but not with ATP, and pyrophosphorolysis of oligonucleotides was observed only when the 3′-terminal nucleotide was UMP.

The formation of a stable complex by the incubation of native DNA and RNA polymerase with purine ribonucleoside triphosphates has been described by several laboratories (Anthony *et al.*, 1966; Stead and Jones, 1967; diMauro *et al.*, 1969). This complex has been called an "initiation complex" and is characterized by its stability to dissociation in the presence of high salt concentrations and its resistance to inhibition by Rifampicin. A stable complex consisting of DNA, RNA polymerase, and RNA had previously been reported by Bremer and Konrad (1964), and the possibility exists that the stability of the "initiation complex," attributed to the binding of the purine ribonucleoside triphosphates, might, in actuality, be due to the synthesis of short RNA chains (Richardson, 1969). This possibility is strengthened by the observation of Sentenac *et al.* (1968) that a stable complex of poly d(A-T), RNA polymerase, and poly r(A-U) can be isolated by gel filtration on Sephadex G-75 when both ATP and UTP are present in the reaction mixture. However, when radioactive ATP is the only substrate present, no radioactivity appears under the poly d(A-T)–enzyme peak. In addition, the work of Khesin *et al.* (1967) has shown that incubation of T2 DNA and RNA polymerase of *Escherichia coli* with all four ribonucleoside triphosphates is necessary to stabilize the enzyme to high temperatures, trypsin, and high concentrations of ammonium chloride. Although ATP alone caused partial stabilization of the enzyme, this was shown to be due to the synthesis of poly A.

Straat and Ts'o (1970) have recently shown that incubation of poly U and RNA polymerase with complementary oligonucleotides did not lead to the formation of an "initiation complex" and did not protect the enzyme against the action of Rifampicin. In these experiments, the effect of a single ribonucleoside triphosphate on the stability of the template–enzyme complex could not be studied, since the incubation of RNA polymerase with poly U and ATP leads to the synthesis of the homopolymer, poly A.

In the present studies, the effects of single ribonucleoside triphosphates, both purine and pyrimidine, as well as complementary dinucleotides on the stability of the DNA–enzyme complex have been investigated using the d(A-T) copolymer as a template and RNA polymerase from *Escherichia coli*. It will be demonstrated that actual formation of a phosphodiester bond is necessary both for the stabilization of the DNA–enzyme complex to dissociation by high salt and to protect the enzyme against inhibition by Rifampicin. In addition, the specificity of the pyrophosphate exchange reaction with this template and enzyme has been investigated.

Methods and Materials

RNA polymerase was prepared from *E. coli* cells by the method of Chamberlin and Berg (1962) and was further purified by gel filtration in the presence of high salt to remove trace amounts of nucleotides. The enzyme was passed over a

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1.5 × 40 cm column of Bio-Gel A-1.5 in the presence of 0.01 M Tris-HCl buffer, pH 7.8, 0.01 M 2-mercaptoethanol, 0.1 mM EDTA, and 1.0 M KCl.

The binding assay was performed as described by Jones and Berg (1966) with slight modification. The reactions were stopped by rapid chilling and the addition of 2 ml of ice-cold washing buffer containing 0.01 M Tris-HCl buffer (pH 7.8), 5 mM 2-mercaptoethanol, and NaCl as indicated in the legends. The reaction mixtures were filtered on Millipore membrane filters (type HA, 2.4 cm diameter), previously soaked in washing buffer, and washed with 60 ml of washing buffer. The filters were dried and counted with a toluene–Omnifluor (Packard Instrument Co.) solution in a liquid scintillation spectrometer.

The assay for pyrophosphate exchange was essentially as described by Krakow and Fronk (1969). The reactions were stopped by the addition of 0.2 ml of 0.1 M EDTA (pH 6.0), 0.1 ml of 0.1 M sodium pyrophosphate (pH 6.0), and 0.5 ml of a 10% suspension of acid-washed, activated charcoal. After the addition of 3 ml of 0.01 M sodium pyrophosphate, the mixtures were filtered on glass filters (Whatman GF/C, 2.4-cm diameter), previously soaked in 0.01 M sodium pyrophosphate, and washed with 50 ml of 0.01 M sodium pyrophosphate. The filters were dried and counted in a liquid scintillation spectrometer. The counting efficiency in the presence of charcoal was approximately 60%.

The polymerization assay was as previously reported (Downey and So, 1970). The standard incubation mixture contained in a final volume of 0.25 ml: 0.08 M Tris-HCl buffer, pH 7.8; 10 mM $MgCl_2$; 4.8 mM 2-mercaptoethanol; 0.08 A_{260} unit of d(A-T) copolymer; 0.04 mM ATP and UTP (one labeled with ^3H); 10 μg of RNA polymerase; and other components as indicated in the legends.

[^3H]ATP and [^3H]UTP were obtained from Schwarz Bio-Research, Inc. Unlabeled ATP and UTP were purchased from either P-L Biochemicals, Inc., or Calbiochem. Unlabeled and ^{14}C-labeled d(A-T) copolymers (1–5 × 10^6 daltons) were obtained from General Biochemicals, and were dialyzed before use against 0.01 M Tris-HCl buffer (pH 7.8)–0.1 M NaCl–1.0 mM EDTA. [^{32}P]PP_i was either purchased from New England Nuclear Corp. or prepared according to Berg (1958). The dinucleotides ApU and UpA were obtained from Sigma Chemical Co. and Rifampicin was obtained from Mann Research Laboratories. *E. coli* B cells were purchased from Grain Processing Corp.

Results

Stabilization of the DNA–RNA Polymerase Complex by RNA Synthesis. The incubation of native DNA with RNA polymerase results in the formation of a DNA–enzyme complex which is retained on a cellulose nitrate membrane filter, whereas the enzyme and DNA, separately, are not retained (Jones and Berg, 1966; Anthony *et al.*, 1966). This complex is readily dissociated, and its formation prevented, by high ionic strength.

In Figure 1, the effect of washing a [^{14}C]poly d(A-T)–enzyme complex with buffers containing increasing concentrations of NaCl is shown. At NaCl concentrations of 0.4 M or higher, the [^{14}C]poly d(A-T)–enzyme complex is completely dissociated, and no radioactivity is retained on the Millipore filter. In contrast, once poly r(A-U) synthesis has taken place, the poly d(A-T)–enzyme–poly r(A-U) complex becomes quite resistant to dissociation by high salt. The addition of poly r(A-U) to the [^{14}C]poly d(A-T)–enzyme complex has no stabilizing effect, indicating that the formation of a stable complex requires the active synthesis of RNA.

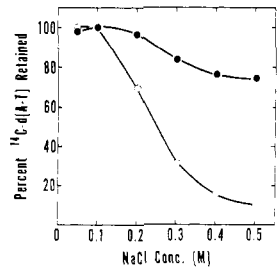

FIGURE 1: Stabilization of the [^{14}C]poly d(A-T)–RNA polymerase complex by poly r(A-U) synthesis. The reaction mixtures were as described in Methods and Materials except that (1) [^{14}C]d(A-T) copolymer, 1.2 μg (8800 cpm/μg) was used; (2) 0.04 M KCl was present in the reaction mixtures; (3) ●—● (incubated with 0.08 mM ATP and UTP); (4) ○—○ (incubated in the absence of ATP and UTP). The reaction mixtures were incubated for 4 min at 25°, and the reactions were stopped by the addition of washing buffer containing the indicated concentration of NaCl.

Requirements for Stabilization of the DNA–RNA Polymerase Complex. Anthony *et al.* (1966) and Stead and Jones (1967) have suggested that the incubation of DNA and enzyme with the initiating ribonucleoside triphosphates ATP and GTP results in the formation of a DNA–enzyme–purine nucleotide complex which is stable to dissociation by high salt. Although no measurable RNA synthesis was observed under these conditions, the possibility that short oligonucleotides were formed has not been ruled out.

When the d(A-T) copolymer is used as a template for the synthesis of poly r(A-U), the synthesis of homopolymers, either poly A or poly U, does not occur. Thus, it is possible to study the effect of a single ribonucleoside triphosphate on the stability of the template–enzyme complex. As shown in Table I, incubation of either ATP or UTP individually with the [^{14}C]d(A-T) copolymer and RNA polymerase does not result in stabilization of the poly d(A-T)–enzyme complex to dissociation by high salt. However, when both ATP and UTP are present together (*i.e.*, where there is synthesis of poly r(A-U)) optimal stabilization of the poly d(A-T)–enzyme complex results, as measured by the membrane filtration method in the presence of 0.4 M NaCl.

Recent studies in this laboratory have shown that the dinucleotides complementary to the d(A-T) copolymer, ApU and UpA, stimulate synthesis of poly r(A-U), as measured by the incorporation of ^3H- or ^{14}C-labeled ribonucleoside triphosphates, while inhibiting initiation with [γ-^{32}P]ATP (Downey and So, 1970). Since RNA chain initiation with the d(A-T) copolymer occurs almost exclusively with ATP (Maitra and Hurwitz, 1965), these studies indicate that, in the presence of a complementary dinucleotide, chain initiation is bypassed in the synthesis of poly r(A-U). This is consistent with the observation of Niyogi and Stevens (1955) that oligonucleotides complementary to the template are incorporated into the 5′ end of the product polynucleotides.

The fact that the dinucleotides cannot be incorporated into internal portions of RNA chains, together with the restriction

TABLE I: Stabilization of the [^{14}C]Poly d(A-T)–Enzyme Complex by the Formation of a Single Phosphodiester Bond.[a]

Components Added	Amount of [^{14}C]Poly d(A-T) Retained on Filter (cpm)
Control	330
ATP	400
ApU	340
ApU, ATP	1300
UTP	450
ATP, UTP	2130
UpA	420
UpA, UTP	1400
ApU, UTP	1300
UpA, ATP	460

[a] The reaction mixtures were as described in Methods and Materials except that (1) [^{14}C]poly d(A-T), 1.2 μg (8800 cpm/μg), was used; (2) 0.08 mM ATP and UTP and 0.32 mM UpA and ApU were added as indicated; and (3) the reaction mixtures also contained 0.04 M KCl and 8 μg of RNA polymerase in a final volume of 0.25 ml. The reaction mixtures were incubated for 2 min at 25°. The washing buffer contained 0.4 M NaCl.

TABLE II: Inhibition of Phosphodiester Bond Formation by Rifampicin.[a]

Components Added	Amount of [^{14}C]Poly d(A-T) Retained on Filter (cpm)
Control	330
ApU, ATP	1490
ApU, ATP, Rifampicin	690
UpA, UTP	1300
UpA, UTP, Rifampicin	560
ATP, UTP	2110
ATP, UTP, Rifampicin	640

[a] The reaction mixtures and incubation conditions were as described in Table I except for the addition of 0.1 μg of Rifampicin where indicated. The control contained only enzyme and [^{14}C]poly d(A-T).

on substrate requirements imposed by the perfectly alternating base sequence of the poly d(A-T) template, makes feasible a study of the formation of a single phosphodiester bond. Incubation of the poly d(A-T)–enzyme complex with ApU and ATP would result in the formation of the trinucleoside diphosphate ApUpA. The absence of UTP in the reaction mixture would prevent further chain elongation. Similarly, UpA and UTP would form the trinucleoside diphosphate UpApU when incubated with the poly d(A-T)–enzyme complex. In this case, the absence of ATP prevents chain elongation.

As can be seen in Table I, neither ApU nor UpA stabilizes the poly d(A-T)–enzyme complex to dissociation by high salt. Stabilization is achieved, however, by the formation of a phosphodiester bond between ApU and ATP (ApUpA) or between UpA and UTP (UpApU). Since there is no possibility for the formation of a phosphodiester bond between UpA and ATP, no stabilization occurs when the poly d(A-T)–enzyme complex is incubated with UpA and ATP. This clearly demonstrates the substrate specificity imposed on the enzyme by the DNA template. Similarly, formation of the trinucleotide ApUpU cannot occur with the d(A-T) copolymer as template. However, a significant stabilization occurs when the poly d(A-T)–enzyme complex is incubated with ApU and UTP. This stabilization appears to be the result of pyrophosphorolysis of the dinucleotide ApU, followed by re-formation of the bond. The evidence supporting this hypothesis will be discussed later.

Inhibition of Phosphodiester Bond Formation by Rifampicin. Rifampicin, a semisynthetic antibiotic, has been shown to inhibit bacterial RNA synthesis by binding to RNA polymerase (Wehrli *et al.*, 1968; diMauro *et al.*, 1969). This antibiotic causes almost complete inhibition when added before the onset of RNA synthesis, but has little effect once synthesis has begun (Sippel and Hartmann, 1968). Rifampicin does not prevent the binding of RNA polymerase to its DNA template (Umezawa *et al.*, 1968); however, it has been suggested that Rifampicin may inhibit chain initiation by preventing the binding of the first ribonucleoside triphosphate to the DNA–enzyme complex (diMauro *et al.*, 1969).

As can be seen in Table II, Rifampicin inhibits internucleotide bond formation between ApU and ATP, UpA and UTP, and the synthesis of poly r(A-U), as measured by the formation of a complex resistant to dissociation by high salt. Preincubation of the poly d(A-T)–enzyme complex with either ATP, UTP, ApU, or UpA does not protect the enzyme against the action of Rifampicin.

Although the poly d(A-T)–enzyme complex can be stabilized by the formation of a phosphodiester bond between either ApU and ATP or UpA and UTP, only the formation of the trinucleotide ApUpA protects the enzyme against inhibition by Rifampicin. Incubation of the poly d(A-T)–enzyme complex with UpA and UTP affords no protection against Rifampicin inhibition. This is shown in Table III where the degree of protection against Rifampicin inhibition is measured by the rate of subsequent poly r(A-U) synthesis when the alternating ribonucleoside triphosphate is added back in the presence of the antibiotic.

Figure 2 shows that the degree of Rifampicin inhibition of poly r(A-U) synthesis is time dependent, being greater at longer incubation periods. The amount of protection afforded by the formation of a single phosphodiester bond is also time dependent, as Rifampicin inhibits the reinitiation of poly r(A-U) chains.

Pyrophosphate Exchange Reaction with UTP. When the d(A-T) copolymer is used as a template, RNA polymerase catalyzes an exchange reaction between PP_i and UTP in the presence of a catalytic amount of ATP (Goldberg *et al.*, 1963; Krakow and Fronk, 1969). It has been suggested that the PP_i exchange reaction catalyzed by RNA polymerase, as well as that catalyzed by DNA polymerase, is the result of pyrophosphorolysis of a newly formed phosphodiester bond (Krakow

TABLE III: Effect of Phosphodiester Bond Formation on Rifampicin Inhibition.[a]

Components Added During Preincubation	Components Added During Subsequent Incubation	Per Cent Activity in the Presence of Rifampicin
	Experiment 1	
None	UpA, [^3H]ATP, UTP	52
UpA	[^3H]ATP, UTP	53
UpA, UTP	[^3H]ATP	59
UpA, ATP	[^3H]UTP	53
	Experiment 2	
None	ApU, ATP, [^3H]UTP	41
ApU	ATP, [^3H]UTP	49
ApU, ATP	[^3H]UTP	94
ApU, UTP	[^3H]ATP	48

[a] The reaction mixtures were as described in Methods and Materials except for the addition of 0.08 mM ApU or UpA. The reaction mixtures were preincubated for 4 min at 37°, followed by the addition of 0.1 μg of Rifampicin and the other components indicated. The reaction mixtures were then incubated for 6 min at 25°. Per cent activity was calculated from control experiments in which no Rifampicin was added during the incubation period. When Rifampicin was present during the preincubation period, 14% activity was observed.

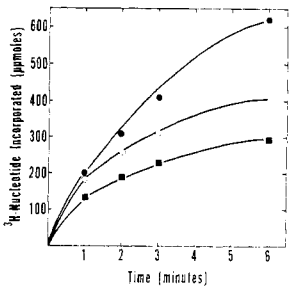

FIGURE 2: The degree of inhibition of RNA synthesis by Rifampicin as a function of time. The reaction mixtures were as described in Methods and Materials except that (1) 0.04 mM ATP and UTP and 0.32 mM ApU were present, and (2) the concentration of the enzyme was 15.2 μg in a final volume of 0.25 ml. The reaction mixtures were preincubated with ApU and ATP (○—○) and without ApU and ATP (■—■) for 4 min at 37°, followed by the addition of 0.1 μg of Rifampicin and [^3H]UTP. The reaction mixtures were further incubated for the times indicated. The control (●—●) was preincubated in the presence of ApU and ATP and incubated in the absence of Rifampicin.

and Fronk, 1969; Deutscher and Kornberg, 1969).

As shown in Table IV, there is little or no PP_i exchange when the poly d(A-T)–enzyme complex is incubated with either ATP, UpA, UpA and ATP, or ApU and ATP, suggesting that no PP_i exchange occurs with ATP. When UTP alone is incubated with the d(A-T) copolymer and RNA polymerase, a sixfold increase in charcoal-adsorbable radioactivity is observed, probably indicating that the enzyme preparation is contaminated with trace amounts of nucleotides. This is consistent with the observation that there is always a low but significant background in the binding assay. Although several purification procedures have been tried, we have not been able to remove all of the nucleotides from our enzyme preparations.

A 30-fold increase in charcoal-adsorbable radioactivity is seen when the poly d(A-T)–enzyme complex is incubated with UpA and UTP, or where there is formation of a single internucleotide bond with a 3′-terminal uridine nucleotide. Incubation of ApU with UTP also results in a significant amount of PP_i exchange, suggesting that pyrophosphorolysis can occur with a preformed phosphodiester bond, followed by re-formation of ApU and repetition of the cycle. These results are consistent with the observation that incubation of the poly d(A-T)–enzyme complex with ApU and UTP results in the formation of a complex resistant to dissociation by high salt. The inability of UpA and ATP to stabilize the poly d(A-T)–enzyme complex in the presence of PP_i would suggest that the pyrophosphorolysis reaction requires a uridine nucleotide in the 3′-terminal position.

As has been reported by Krakow and Fronk (1969) with RNA polymerase from *M. luteus*, high concentrations of ATP inhibit the PP_i exchange reaction when the d(A-T) copolymer is the template. This may be due to: (1) competition between the polymerization and exchange reactions for UTP; (2) the specificity of the pyrophosphorolysis reaction for a 3′-terminal uridine nucleotide; and (3) the absence of PP_i exchange with ATP.

Discussion

In continuing our studies on the mechanism of RNA synthesis, an attempt has been made to characterize the DNA–

TABLE IV: Requirements for Pyrophosphate Exchange.[a]

Additions	PP_i Incorporated (μμmoles)
None	70
ATP	110
UTP	380
ATP (0.32 mM), UTP	440
ATP (0.08 mM), UTP	1490
ApU	100
ApU, ATP	110
ApU, UTP	690
UpA	130
UpA, UTP	2150
UpA, ATP	90

[a] The reaction mixtures were as described in Methods and Materials except that (1) the concentrations of ATP, UTP, ApU, and UpA were 0.32 mM unless otherwise indicated, and (2) [^{32}P]PP_i, 1.0 mM (1.0×10^6 cpm/μmole), was included in the reaction mixture. The reaction mixtures were preincubated in the absence of ATP or UTP and [^{32}P]PP_i for 5 min at 37°, followed by incubation at 37° for 10 min.

enzyme complex before and after the formation of a single phosphodiester bond. We have studied the effects of bond formation on the stability of the DNA–enzyme complex to dissociation by high salt concentrations and on the sensitivity of the enzyme to the inhibitory action of Rifampicin.

The use of poly d(A-T), a double-helical, alternating copolymer of deoxyadenylate and thymidylate, as template for the RNA polymerase of *E. coli*, allows one to study the effects of the formation of a single phosphodiester bond on the template–enzyme complex.

Incubation of the poly d(A-T)–enzyme complex with ApU and ATP or UpA and UTP results in the formation of the trinucleoside diphosphates ApUpA and UpApU, respectively. Further elongation of the poly r(A-U) chain does not occur until the alternating ribonucleoside triphosphate is subsequently added to the reaction mixture.

It is clear from the results presented that incubation of the poly d(A-T)–enzyme complex with a single ribonucleoside triphosphate, either ATP or UTP, does not lead to the formation of a complex stable to dissociation by high salt, nor does it afford protection from Rifampicin inhibition.

Anthony *et al.* (1966) and Stead and Jones (1967) have reported that the incubation of a DNA–enzyme complex with the initiating ribonucleoside triphosphates ATP and GTP results in the formation of an "initiation complex" which is stable at high ionic strength. Furthermore, the formation of such a complex renders the enzyme resistant to inhibition by Rifampicin (Sippel and Hartman, 1968; diMauro *et al.*, 1969; Straat and Ts'o 1970). Although measurable RNA synthesis was not detected in these studies, it is possible that the observed stabilization was the result of synthesis of short oligonucleotides, rather than the effect of initiating purine ribonucleoside triphosphates, as pointed out by Richardson (1969). The studies of Khesin *et al.* (1967) further strengthen this possibility. These authors have shown that incubation of the DNA–enzyme complex with initiating ribonucleoside triphosphates alone is not sufficient to stabilize RNA polymerase, and that the partial stabilization effected by incubation with ATP is the result of poly A synthesis. In addition, Sentenac *et al.* (1968) have shown that single ribonucleoside triphosphates do not bind to the DNA–enzyme complex in the absence of RNA synthesis.

The complementary dinucleotides ApU and UpA are also ineffective in stabilizing the poly d(A-T)–enzyme complex to dissociation by high salt and in protecting the enzyme from Rifampicin inhibition. This result is consistent with the observation of Straat and Ts'o (1970) that, in poly U directed synthesis of poly A with RNA polymerase of *M. luteus*, no protection against Rifampicin inhibition was observed when the poly U–enzyme complex was incubated with adenine oligonucleotides.

The enzyme-catalyzed synthesis of a single phosphodiester bond, either between ApU and ATP to form the trinucleoside diphosphate ApUpA, or between UpA and UTP to form UpApU, results in the formation of a poly d(A-T)–enzyme–trinucleotide complex which is stable at high salt concentrations. The formation of the trinucleotide ApUpA also protects the enzyme against the inhibitory action of Rifampicin and renders the subsequent synthesis of poly r(A-U) possible in the presence of this antibiotic. The synthesis of UpApU, however, does not protect the enzyme against Rifampicin inhibition. Furthermore, the enzyme catalyzes a PP_i exchange reaction with UTP in the presence of UpApU but not with ATP in the presence of ApUpA.

It has further been observed that incubation of the poly d(A-T)–enzyme complex with ApU and UTP results in both stabilization of the poly d(A-T)–enzyme complex to the effects of high salt concentrations, as well as considerable PP_i exchange. As the trinucleotide ApUpU cannot be formed when poly d(A-T) is used as template, the simplest explanation for the observed results is an initial removal of the 3'-UMP by PP_i followed by the incorporation of UTP to re-form the dinucleotide ApU. That neither stabilization of the poly d(A-T)–enzyme complex nor PP_i exchange is observed in the presence of UpA and ATP appears to be the result of the specificity of the pyrophosphorolysis reaction for a 3'-terminal uridine nucleotide. It is of interest that PP_i exchange with ATP is observed with RNA polymerase when poly dT is used as template (Krakow and Fronk, 1969), suggesting that the secondary structure of the template plays an important role in determining the activity as well as the substrate specificity of the enzyme.

The studies with Rifampicin indicate that this antibiotic inhibits the formation of the first phosphodiester bond, and not any prior step in RNA synthesis. It is interesting to note that the formation of a trinucleotide that does not allow pyrophosphorolysis to occur (ApUpA) protects the enzyme from inhibition by Rifampicin, while the formation of a trinucleotide that readily undergoes pyrophosphorolysis (UpApU) offers no such protection. Whether or not there is a causal relationship between these two phenomena is not, as yet, clear. Perhaps during the process of pyrophosphorolysis the enzyme is left unprotected and available to attack by the antibiotic. Alternatively, it is possible that the binding of ApU and ATP and subsequent formation of the trinucleotide ApUpA distort the Rifampicin binding site so that it no longer is able to form a complex with the antibiotic, while the binding of UpA and UTP and the formation of UpApU have no effect on the Rifampicin-binding site. A third possibility would be that the three-dimensional structures of the poly d(A-T)–enzyme–trinucleotide complexes are significantly different, so that the binding of ApUpA masks the Rifampicin binding site while the binding of UpApU does not.

Recent results published by Lill *et al.* (1970) have indicated that preincubation of RNA polymerase with poly d(A-T) alone rendered the enzyme partially resistant to Rifampicin inhibition. We have confirmed the results of Lill *et al.* and find that the formation of a phosphodiester bond between ApU and ATP (ApUpA) increases the resistance of the enzyme to the effects of Rifampicin over and above that observed in the presence of poly d(A-T) alone. Under our assay conditions, preincubation of RNA polymerase with poly d(A-T) alone, with poly d(A-T) and ApU, or with poly d(A-T) and ATP resulted in approximately 40% protection of the enzyme whereas preincubation of the enzyme with poly d(A-T), ApU, and ATP resulted in 80% protection. The possibility that this partial protection is due to contamination of the enzyme with trace amounts of ribonucleoside triphosphates has not been ruled out.

Acknowledgment

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References

Anthony, D. D., Zeszotek, E., and Goldthwait, D. A. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1026.
Berg, P. (1958), *J. Biol. Chem. 233*, 601.
Bremer, H., and Konrad, M. W. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 801.
Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 81.
Deutscher, M. P., and Kornberg, A. (1969), *J. Biol. Chem. 244*, 3019.
diMauro, E., Snyder, L., Marino, P., Lamberti, A., Coppo, A., and Tocchini-Valentini, G. P. (1969), *Nature (London) 222*, 533.
Downey, K. M., and So, A. G. (1970), *Biochemistry 9*, 2520.
Goldberg, I. H., Rabinowitz, M., and Reich, E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 226.
Jones, O. W., and Berg, P. (1966), *J. Mol. Biol. 22*, 199.
Khesin, R. B., Astaurova, O. B., Shemyakin, M. F., Kamzolova, S. G. and Manyakov, V. F. (1967), *Mol. Biol. 1*, 617.
Krakow, J. S., and Fronk, E. (1969), *J. Biol. Chem. 244*, 5988.
Lill, H., Lill, U., Sippl, A., and Hartmann, G. (1970), *in* Proceedings of the First Small Colloquium on RNA Polymerase and Transcription, Silvester, L., Ed., Amsterdam, North-Holland.
Maitra, U., and Hurwitz, J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 815.
Niyogi, S. K., and Stevens, A. (1965), *J. Biol. Chem. 240*, 2593.
Richardson, J. P. (1969), *Progr. Nucleic Acid Res. Mol. Biol. 9*, 75.
Sentenac, A., Simon, E. J., and Fromageot, P. (1968), *Biochim. Biophys. Acta 161*, 299.
Sippel, A., and Hartmann, G. (1968), *Biochim. Biophys. Acta 157*, 218.
Stead, N. W., and Jones, O. W. (1967), *Biochim. Biophys. Acta 145*, 679.
Straat, P. A., and Ts'o, P. O. P. (1970), *Biochemistry 9*, 926.
Umezawa, H., Mizuno, S., Yamazaki, H., and Nitta, K. (1968), *J. Antibiot. 21*, 234.
Wehrli, W., Knusel, F., Schmid, K., and Staehlin, M. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 667.

Nicking Activity of an Endonuclease I–Transfer Ribonucleic Acid Complex of *Escherichia coli**

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ABSTRACT: An endonuclease I-tRNA complex, extracted from *Escherichia coli* JC411, but not present to any appreciable extent in an endonuclease I deficient *E. coli* mutant strain, was shown to catalyze in the presence of 0.5 M NaCl an average of a single nick per covalently closed circular DNA molecule of colicinogenic factor E_1 and the RFI form of ϕX-174.

A similar activity is shown by purified endonuclease I in the presence of *E. coli* tRNA and 0.5 M NaCl. *E. coli* tRNA forms a stable complex with endonuclease I that exhibits a sedimentation coefficient ($s^0_{20,w}$) of 6.0. Purified endonuclease I in the absence of tRNA exhibited an $s^0_{20,w}$ of 3.2 in sucrose gradient sedimentation velocity studies and characteristically degraded DNA to acid soluble oligonucleotides. The nicking activity of the endonuclease I–tRNA complex in the presence of 0.5 M NaCl requires Mg^{2+} and is inhibited by EDTA. With the RFI form of ϕX-174 the nick catalyzed by the endonuclease I–tRNA complex is not strand specific, but occurs with similar frequency in each of the complementary strands.

Endonuclease I of *Escherichia coli*, isolated and purified by Lehman *et al.* (1962), has been shown to catalyze the endonucleolytic cleavage of DNA to acid-soluble oligonucleotides of an average chain length of 7 nucleotides. Studies on the mechanism of degradation of DNA by this enzyme have revealed that the enzyme degrades DNA principally according to single hit kinetics (double strand scission, or chopping) (Cordomier and Bernardi, 1965; Studier, 1965). *Escherichia coli* endonuclease I has also been reported to be strongly inhibited by tRNA (Lehman *et al.*, 1962; Hurwitz *et al.*, 1965).

In this report the formation of a tRNA–endonuclease I complex that sediments more rapidly than unassociated endonuclease I in a sucrose gradient is described. The tRNA-associated endonuclease I exhibits limited endonuclease activity that occurs principally according to double hit kinetics (single strand scission, nicking reaction) in the presence of a high concentration of sodium chloride.

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