Nucleic Acid Enzymology of Extremely Halophilic Bacteria

HALOBACTERIUM CUTIRUBRUM DEOXYRIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE

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(Received 1 September 1970)

1. DNA-dependent RNA polymerase was purified 150-fold from crude extracts of the extreme halophile *Halobacterium cutirubrum*. 2. The enzyme requires the presence of native DNA and all four nucleoside triphosphates to incorporate ^{14}C -labelled nucleoside triphosphate into an acid-insoluble ribonuclease-sensitive product. 3. It has an absolute requirement for both Mn^{2+} and Mg^{2+} . 4. The polymerase requires a high salt concentration for stability, but is markedly inhibited by univalent cations. 5. Its molecular weight is very low compared with that of *Escherichia coli* RNA polymerase.

Despite the unusual and interesting properties of the enzymes of extremely halophilic bacteria (Larsen, 1967; Kushner, 1968), no studies of the nucleic acid enzymology of these organisms had been published until the recent preliminary reports of the isolation of polynucleotide phosphorylase (Peterkin & Fitt, 1970) and DNA-dependent and RNA-dependent RNA polymerases (Fitt & Louis, 1970) from *Halobacterium cutirubrum*. The DNA-dependent RNA polymerase [nucleoside triphosphate-RNA nucleotidyltransferase (DNA-dependent); EC 2.7.7.6] was of particular interest because of the established importance of such enzymes in normal cellular RNA synthesis (Richardson, 1969; Seifert & Zillig, 1969; Burgess & Travers, 1970). In addition, it seemed possible that the *H. cutirubrum* enzyme might prove of value in the study of the mechanism of the template-substrate-enzyme interaction in limiting conditions of ionic strength if its salt requirements proved similar to those of previously studied enzymes of this bacterium.

In the present paper we describe the partial purification and preliminary study of the properties of *H. cutirubrum* DNA-dependent RNA polymerase. Further studies have also been made of the polynucleotide phosphorylase (Peterkin & Fitt, 1971) and the RNA-dependent RNA polymerase (Louis & Fitt, 1971) of this organism and the approximate molecular weights of all three enzymes have been determined by gel filtration and density-gradient centrifugation (Louis, Peterkin & Fitt, 1971).

EXPERIMENTAL

Materials. Intermediates and enzymes were purchased from the following suppliers: ^{14}C - and ^3H -labelled nucleoside triphosphates, Amersham/Searle Corp., Don Mills, Ont., Canada; Bio-Gel P-60 polyacrylamide gel, Bio-Rad Laboratories, Richmond, Calif., U.S.A.; high-molecular-weight wheat-germ RNA, Calbiochem, Los Angeles, Calif., U.S.A.; Blue Dextran and Ficoll, Pharmacia (Canada) Ltd., Montreal, P.Q., Canada; nucleoside triphosphates, P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; adenosine and calf thymus DNA, Sigma Chemical Co., St Louis, Mo., U.S.A.; crystalline pancreatic deoxyribonuclease and ribonuclease, Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Cell culture. *H. cutirubrum* strain no. 9 cells were grown and harvested as described by Peterkin & Fitt (1971).

Preparation of *H. cutirubrum* DNA-dependent RNA polymerase. All operations were carried out at 0–5°C.

The freshly harvested cells (24g wet wt.) were stirred with 2.5M-KCl–1M-NaCl–10mM-tris-HCl buffer, pH 8.6 ('high-salt buffer'), about 3ml/g, for about 1 h to give about 90ml of homogeneous suspension. Portions (30ml) of the suspension were sonicated for 30s in a Bronwill Biosonik II apparatus set at 70% of maximum output. The suspension was centrifuged for 30 min at 27000g to remove cell debris, and the supernatant was then centrifuged for 2 h at 78000g in a preparative ultracentrifuge. The pellet was discarded and the supernatant (crude extract, Table 1) was used for preparation of both the DNA-dependent and RNA-dependent RNA polymerases.

The crude extract was adjusted to pH 4 with 1M-HCl and the suspension was centrifuged immediately for 10 min at 27000g. The RNA-dependent RNA polymerase was precipitated completely, leaving the DNA-dependent

enzyme in the supernatant. The pH of the latter was returned to 8.6 without delay by addition of 1M-NaOH. The solution was dialysed overnight against high-salt buffer (50 vol.) and then concentrated to about 10 ml by dialysis against 50% (w/v) Ficoll in high-salt buffer. The enzyme was then purified by upward-flow polyacrylamide gel filtration in high-salt buffer on a column (2.5 cm $\times$ 85 cm) of Bio-Gel P-60 prepared and run as described by Louis *et al.* (1971). The E_{280} of the effluent was monitored continuously (LKB Uvicord II) and 4 ml fractions were collected. The active fractions were concentrated by dialysis against 50% (w/v) Ficoll in high-salt buffer. The void and internal volumes of the Bio-Gel P-60 columns were determined just before use with Blue Dextran and adenosine (Louis *et al.* 1971).

Enzyme assays. (a) *H. cutirubrum* DNA-dependent RNA polymerase was assayed by measuring the incorporation of ^{14}C -labelled nucleoside triphosphate into an acid-insoluble product in the presence of ATP, GTP, UTP, CTP and a DNA template (Hurwitz, Furth, Anders, Ortiz & August, 1961; Krakow & Ochoa, 1963; Nakamoto, Fox & Weiss, 1964; Stevens, 1961). The incubated samples were processed by a modification of the Bollum paper-disc technique (Bollum, 1959). The standard assay medium (0.1 ml) contained: tris-HCl buffer, pH 8.6, 10 μmol ; one ^{14}C -labelled nucleoside triphosphate, 5 nmol (about 10000 d.p.m./nmol); the other three non-radioactive nucleoside triphosphates, 15 nmol each; native calf thymus DNA, 30 μg ; MgCl_2 , 10 μmol ; MnCl_2 , 1 μmol ; enzyme, up to 2.6 units. After incubation at 37°C for 1 h, a sample (up to 85 μl) of the mixture was streaked on a numbered 2 cm $\times$ 3 cm strip of Whatman no. 3 filter paper. The strips were collected in cold 7% (w/v) HClO_4 (10 ml/strip) and the suspension was swirled (New Brunswick Scientific Co. rotary shaker) for at least 7 min. The strips were allowed to settle and the fluid was removed with an aspirator. The washing procedure was repeated successively with similar volumes of cold 7% (w/v) HClO_4 , 1% (w/v) HClO_4 and ethanol. The washed strips were air-dried, transferred to individual vials containing 0.5% (w/v) 2,5-diphenyloxazole in toluene (10 ml) and their radioactivities counted to 2% accuracy in a Beckman LS-133 liquid-scintillation counter. All the results were corrected for quenching (see below). Control experiments without enzyme were performed and their radioactivity was subtracted from the assay values. To determine the specific radioactivity of radioactive nucleoside triphosphate, samples (10 μl) of the stock nucleoside triphosphate solution (0.5 $\mu\text{mol}/\text{ml}$) were streaked on filter-paper strips and the strips were dried under an i.r. lamp and their radioactivities counted as described above. A unit was defined as the amount of enzyme catalysing incorporation of 1 nmol of [^{14}C]ATP into acid-insoluble material/h.

(b) Nuclease assays. Deoxyribonuclease and ribonuclease activities in samples of *H. cutirubrum* DNA-dependent RNA polymerase were determined by measuring the liberation of acid-soluble products from the appropriate nucleic acid. The assay medium was that described above for the polymerase assay with the following modifications: ^{14}C -labelled nucleoside triphosphate and non-radioactive nucleoside triphosphates were omitted and either DNA (30 μg ; deoxyribonuclease assay) or RNA (30 μg ; ribonuclease assay) was included. The

assays were performed both with and without 1.5M-KCl-0.6M-NaCl in the medium. After 1 h at 37°C, the reaction was stopped with an equal volume of 0.5% (w/v) uranyl acetate in 7% (w/v) HClO_4 . The mixtures were kept at 0°C for 10 min. Carrier nucleic acid (10 μg ; DNA or RNA, as appropriate) was added and the suspensions were kept at 0°C for a further 5 min. They were then diluted to 1 ml with water and centrifuged. The E_{260} of the supernatant was measured in a Zeiss PMQ II spectrophotometer, with a similarly treated incubation mixture that lacked the substrate as blank. (Controls without enzyme were also always performed and treated as above. No hydrolysis of the nucleic acid occurred in these control incubations.)

Correction of radioactivity assay results for quenching. All results of radioactivity assays with either ^{14}C - or ^3H -labelled nucleoside triphosphate were corrected to d.p.m. as follows. Quench-correction curves were obtained by using quenched standards (New England Nuclear Corp., Boston, Mass., U.S.A.) and the external standardization system of the Beckman LS-133 liquid-scintillation counter. The curves were nearly linear in the range used, so straight lines were fitted to them by the method of least squares; the limits between which the efficiency for a given standardization ratio was within 1% of the straight-line values were then determined visually. This information was used to program an Olivetti-Underwood Programma 101 desk-top computer to correct c.p.m. to d.p.m. (The program automatically rejects values outside the usable range of the straight-line approximation. All efficiencies in our work have been within this range.)

Protein assay. Protein was determined spectrophotometrically (Warburg & Christian, 1942).

RESULTS AND DISCUSSION

Purification of *H. cutirubrum* DNA-dependent RNA polymerase. The results of a typical purification of the enzyme are shown in Table 1. The preparation must be completed as rapidly as possible, so all fractions are initially assayed without prior removal of salt to avoid delay. Since the salt concentration is the same throughout the preparation, and the extent of inhibition by salt (see below) is unaffected by the purity of the enzyme, this permits a rapid check on the relative purification and yield. The absolute activities are then determined as soon as convenient, or may be calculated by using a correction factor. The specific enzyme activity of the crude extract varies from one preparation to another, but the overall purification achieved has always been about 150-fold. The purified Bio-Gel P-60 fraction is free from nucleases active in the conditions of the standard assay, although the crude extract has both deoxyribonuclease and ribonuclease activity.

The DNA-dependent RNA polymerase activity of the crude extract of *H. cutirubrum* is quite low compared with that of extracts from (e.g.) *Escherichia coli*, but recent preliminary experiments suggest

Table 1. Purification of *H. cutirubrum* DNA-dependent RNA polymerase

The enzyme was purified as described in the Experimental section and activity was determined by using the standard assay. Numbers in parentheses represent units of RNA-dependent RNA polymerase activity determined as described by Louis & Fitt (1971).

Fraction	Activity (units)		Sp. activity (units/mg)		Relative purification	Yield (%)
	Without salt	With salt	Without salt	With salt		
Crude extract	2875	180 (108)	3.2	0.2	1	100
pH 4 supernatant	994	62.2 (0)	13.1	4.1	20.5	34.6
Bio-Gel P-60	361	22.6 (0)	493.0	30.9	154	12.5

Table 2. Template requirement of *H. cutirubrum* DNA-dependent RNA polymerase

Enzymic activity was determined in the standard assay with and without 1.5M-KCl-0.6M-NaCl with the indicated nucleic acid as template. The sonicated DNA was prepared by sonication for 30s of native calf thymus DNA (2mg/ml) with a Bronwill Biosonik III apparatus set at 40% of maximum intensity. To obtain heat-denatured DNA, a solution of native calf thymus DNA (2mg/ml) was heated at 100°C for 10min and then cooled in ice.

Template	ATP incorporated (nmol)	
	Without salt	With salt
None	0	0
Native DNA (300 µg/ml)	1.02	0.10
Sonicated DNA (300 µg/ml)	1.08	0.08
Heat-denatured DNA (300 µg/ml)	0.04	0
High-mol.-wt. RNA (750 µg/ml)	0.01	0

Fig. 1. Effect of DNA concentration on activity of *H. cutirubrum* DNA-dependent RNA polymerase. The activity was determined in the standard assay with the indicated concentration of native calf thymus DNA.

that this may be due, at least in part, to the inefficiency of the method of extraction used. Also, the mean generation time of *H. cutirubrum* is some 30 times that of *E. coli* (Kushner, 1968) and the relative activity in these two bacteria may reflect this difference.

Enzymic properties of *H. cutirubrum* DNA-dependent RNA polymerase. The purified enzyme had an absolute requirement for native DNA as template. The results in Table 2 show that in the standard assay conditions neither heat-denatured DNA nor high-molecular-weight RNA stimulated incorporation of [¹⁴C]ATP into an acid-insoluble form. Sonication of native calf thymus DNA had no significant effect on its template activity. Fig. 1 shows the effect of changes in template concentration on the activity of the enzyme in the standard assay conditions: a plateau is reached at a DNA concentration of about 300 µg/ml. It should be emphasized that after the pH 4 fractionation, the DNA-dependent polymerase

is totally devoid of RNA-dependent activity, in marked contrast to the RNA polymerases of *Micrococcus lysodeikticus* (Fox, Robinson, Haselkorn & Weiss, 1964), *E. coli* (Gomatos, Krug & Tamm, 1964) and *Azotobacter vinelandii* (Krakow & Ochoa, 1963), which catalyse both DNA- and RNA-directed synthesis of RNA. The RNA-dependent RNA polymerase activity of *H. cutirubrum* appears to be associated entirely with a different enzyme (Fitt & Louis, 1970; Louis & Fitt, 1971) that is easily separable from the DNA-dependent polymerase described in the present paper.

Similar results to those described above were obtained with 1.5M-potassium chloride-0.6M-sodium chloride in the assay medium, although the absolute amounts of incorporation were then lower than in the absence of salt.

The polymerase required the presence of all four ribonucleoside triphosphates (Table 3). Omission of any one decreased the incorporation by at least 90%. In the presence of a single nucleoside triphosphate no incorporation took place, except to a

Table 3. *Substrate requirements of H. cutirubrum DNA-dependent RNA polymerase*

Enzymic activity of the purified enzyme was determined by the standard assay with the indicated nucleotides (150 nmol/ml in all cases, including the radioactive nucleotide) as substrates.

Nucleotides	Radioactive nucleotide	¹⁴ C- or ³ H-labelled nucleotide incorporated (nmol)
(1) ATP, CTP, UTP, GTP	[¹⁴ C]ATP	1.11
(2) CTP, UTP, GTP	[³ H]CTP	0
(3) ATP, UTP, GTP	[¹⁴ C]ATP	0.08
(4) ATP, CTP, GTP	[³ H]CTP	0.10
(5) ATP, CTP, UTP	[¹⁴ C]ATP	0.08
(6) ATP, CTP	[¹⁴ C]ATP	0
(7) ATP, UTP	[¹⁴ C]ATP	0.09
(8) ATP, GTP	[¹⁴ C]ATP	0
(9) CTP, UTP	[³ H]UTP	0.05
(10) CTP, GTP	[³ H]CTP	0
(11) UTP, GTP	[³ H]UTP	0.01
(12) ATP	[¹⁴ C]ATP	0
(13) CTP	[³ H]CTP	0.06
(14) UTP	[³ H]UTP	0
(15) GTP	[³ H]GTP	0

limited extent with CTP. It is therefore possible that the Bio-Gel P-60 fraction contained a small amount of CTP polymerase, but other nucleoside triphosphate polymerases active in these conditions were absent. The enzyme had a sharp substrate optimum of 150 nmol of each nucleoside triphosphate/ml, similar to that of the RNA-dependent RNA polymerase of *H. cutirubrum* (Louis & Fitt, 1971). However, in the standard assay system the [¹⁴C]ATP had three times the specific radioactivity used in the experiments described in Fig. 2, and it was used at a concentration of 50 nmol/ml together with 150 nmol of the other three nucleoside triphosphates/ml: since in these conditions the activity was only slightly decreased, the sensitivity of the assay was greatly improved.

H. cutirubrum DNA-dependent RNA polymerase has an absolute requirement for both Mg²⁺ and Mn²⁺ in the assay medium (Figs. 3a and 3b). If either one or both cations were omitted no incorporation took place. In the presence of 100 μmol of magnesium chloride/ml there was a pronounced [Mn²⁺] optimum between 8 and 10 μmol/ml (Fig. 3a). However, the activity increased linearly with increasing [Mg²⁺] in the range tested when a [Mn²⁺] of 10 μmol/ml was used. Higher concentrations of Mg²⁺ could not be tried in the presence of the optimum concentration of Mn²⁺, because they led to the formation of brown precipitates and high variable control values.

The enzyme has a sharp pH optimum at 8.6 and its stability during storage does not appear to vary in the range pH 7.5–10.

Fig. 2. Substrate-saturation curve for *H. cutirubrum* DNA-dependent RNA polymerase. The activity of the enzyme was determined by the standard assay with the indicated concentration of nucleoside triphosphate (all four nucleoside triphosphates being present at the concentration shown). Each point represents the average of two determinations that differed by less than 10%.

A thiol such as β-mercaptoethanol or dithiothreitol is frequently included in assay systems for RNA polymerase (cf. Frederick, Maitra & Hurwitz, 1969; Lee-Huang & Warner, 1969), but neither the activity nor the stability of the *H. cutirubrum*

Fig. 3. Effect of changes in (a) Mn^{2+} concentration in the presence of $MgCl_2$ ($100\mu\text{mol/ml}$) and (b) Mg^{2+} concentration in the presence of $MnCl_2$ ($10\mu\text{mol/ml}$) on the activity of *H. cutirubrum* DNA-dependent RNA polymerase in the standard assay. Each point represents the average of two results that differed by less than 10%.

enzyme was affected by the presence of β -mercaptoethanol in concentrations up to 10mM.

Fig. 4 shows the time-course of the reaction in the standard assay conditions. The curve is sigmoidal and there is an appreciable lag phase, possibly due to inefficient chain initiation in these conditions. In all our experiments so far we have used calf thymus DNA as template and it will be interesting to see if the reaction is linear in the presence of *H. cutirubrum* DNA.

The results in Table 4 show that addition of pancreatic deoxyribonuclease to the incubation mixture stops the reaction, presumably through hydrolysis of the DNA template. However, although the product of the reaction was not deoxyribonuclease-sensitive, pancreatic ribonuclease destroyed it and decreased the measured incorporation to a low value. These are the results expected for an enzyme that is DNA-dependent and catalyses formation of a polyribonucleotide. Together with the information on the template specificity (Table 2) and substrate requirements (Table 3) of the enzyme, they prove that it is a DNA-dependent RNA polymerase.

Effect of salts on the stability and activity of H. cutirubrum DNA-dependent RNA polymerase. The enzyme requires a high concentration of potassium chloride or sodium chloride or of both for stability. During storage at 4°C in the high-salt buffer containing 2.5M-potassium chloride and 1M-sodium chloride, it loses 50% of its original activity within 5 weeks. If the potassium chloride and sodium chloride are removed by dialysis against salt-free buffer, the enzyme loses 95% of its activity irreversibly in 24h and is totally inactivated after 48h. Thus it is in this respect a typical example of an enzyme from an extreme halophile (Larsen, 1967; Kushner, 1968).

Fig. 4. Time-course of the reaction. A separate standard assay was performed for each point. The assay tubes were stoppered and toluene was added to prevent microbial contamination. Each point represents the average of two determinations differing by less than 10%.

Table 4. Effect of nucleases on polymerization of ribonucleoside triphosphates by *H. cutirubrum* DNA-dependent RNA polymerase

Standard assay mixtures containing the purified enzyme were incubated for 60 min at 37°C . Water, deoxyribonuclease or ribonuclease were then added as indicated. The 60 min control was prepared immediately by the usual method and the remaining three solutions were incubated for a further 30 min before being processed.

Addition after 60 min	$[^{14}\text{C}]\text{ATP}$ incorporated (nmol)
(1) Water, $20\mu\text{l}$ (60 min control)	1.25
(2) Water, $20\mu\text{l}$ (90 min control)	2.20
(3) Deoxyribonuclease (0.5 mg/ml), $20\mu\text{l}$	1.44
(4) Ribonuclease (0.5 mg/ml), $20\mu\text{l}$	0.18

However, *H. cutirubrum* DNA-dependent RNA polymerase has the quite unexpected property of being partly inhibited by univalent cations. As shown in Fig. 5, potassium chloride at concentrations of 0.5M or higher inhibits the polymerization by about 95%, and sodium chloride at similar concentrations inhibits it by 90%. The extent of inhibition is not affected by the purity of the enzyme. Assay of the purified enzyme for nucleases both in the presence and in the absence of salt showed that none was present, so the inhibition is

Fig. 5. Effect of KCl and NaCl on the activity of *H. cutirubrum* DNA-dependent RNA polymerase. Enzymic activity was determined in the standard assay at the indicated salt concentrations. Each point represents the average of two values that differed by less than 10%. ▲, KCl; ■, NaCl.

not an apparent one due to contamination of the enzyme by deoxyribonucleases or ribonucleases active only in the presence of salt. It is also unlikely that the inhibition is caused by some gross effect on the template in the presence of high concentrations of potassium chloride and sodium chloride. Standard assay mixtures lacking enzyme and nucleotides, but containing the usual amount of template DNA, were prepared with and without 1.5M-potassium chloride–0.6M-sodium chloride. They had identical extinctions at 260nm both before and after centrifugation, and the extinction of the solution containing the salt was unchanged after incubation at 37°C for 1h. It will be interesting to see whether a similar inhibition by salt occurs when *H. cutirubrum* DNA is used as template instead of calf thymus DNA.

All the enzymes previously isolated from extreme halophiles have required at least 2M salt for optimum activity (Larsen, 1967; Kushner, 1968), including *H. cutirubrum* polynucleotide phosphorylase (Peterkin & Fitt, 1970, 1971). Further, the DNA-dependent RNA polymerase is even 30% inhibited by 0.2M-potassium chloride, which has been found to stimulate initiation of polymerization by *E. coli* RNA polymerase (Maitra & Barash, 1969).

Nevertheless, it appears that enzymes inhibited by salt exist in extremely halophilic bacteria: in addition to the DNA-dependent RNA polymerase, a salt-inactivated fatty-acyl synthetase has recently been found in *H. cutirubrum* (M. Kates, personal communication). It is possible that the slow growth rate of *H. cutirubrum* (mean generation time 7–15h; Kushner, 1968) is related to the low activity of the RNA polymerase in a high-salt environment.

Ammonium sulphate has effects similar to those of potassium chloride and sodium chloride on the activity and stability of the enzyme.

Molecular weight of *H. cutirubrum* DNA-dependent RNA polymerase. The approximate molecular weight of the purified enzyme was determined by gel filtration and sucrose-density-gradient centrifugation (Louis *et al.* 1971) and found to be 18000. The smallest functional unit of *E. coli* RNA polymerase, the so-called 'active particle' (Seifert & Zillig, 1969) or 'minimal enzyme' (Burgess, 1969), has sedimentation coefficient 13S and mol.wt. 360000–400000 (Richardson, 1966; Preiss & Zillig, 1967; Maitra & Hurwitz, 1967; Berg, Barrett, Hinkle, McGrath & Chamberlin, 1969). The 'minimal enzyme', which lacks the initiation or σ -factor (Burgess, Travers, Dunn & Bautz, 1969), has been separated (Burgess, 1969) into the following inactive subunits: α , mol.wt. 39000 $\pm$ 5%; β , mol.wt. 155000 $\pm$ 10%; β' , mol.wt. 165000 $\pm$ 10% (mol.wt. of 'minimal enzyme', $\alpha_2\beta\beta'$, 400000 $\pm$ 10%). Thus the *H. cutirubrum* DNA-dependent RNA polymerase is approximately one-twentieth the size of the active *E. coli* enzyme: if further studies substantiate this result, the enzyme should prove of great value in the study of the structural basis of RNA polymerase action.

We thank the Medical Research Council of Canada for an operating grant (to P.S.F.) and the Province of Ontario for a Graduate Fellowship (to B.G.L.).

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