Nucleic Acid Enzymology of Extremely Halophilic Bacteria

HALOBACTERIUM CUTIRUBRUM RIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASE

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1. Crude extracts of the extreme halophile *Halobacterium cutirubrum* contain separable DNA-dependent and RNA-dependent RNA polymerases. 2. The RNA-dependent enzyme has been purified about 2800-fold. 3. It requires RNA, preferably of high molecular weight, and all four ribonucleoside triphosphates to incorporate ^{14}C -labelled nucleoside triphosphate into an acid-insoluble, ribonuclease-sensitive product. 4. Both the stability and activity of the RNA polymerase are relatively insensitive to changes in potassium chloride or sodium chloride concentration, but incorporation is stimulated by both Mg^{2+} and Mn^{2+} . 5. The molecular weight of the enzyme is about 17000–18000.

RNA-directed synthesis of RNA is catalysed by two types of enzymes: (i) DNA-dependent RNA polymerase [nucleoside triphosphate–RNA nucleotidyltransferase (DNA-dependent); EC 2.7.7.6], from e.g. *Escherichia coli*, *Micrococcus lysodeikticus* and *Azotobacter vinelandii*, which will copy RNA as well as DNA templates, although its activity is much less with the former (Krakow & Ochoa, 1963; Fox, Robinson, Haselkorn & Weiss, 1964; Gomatos, Krug & Tamm, 1964), and (ii) exclusively RNA-dependent RNA polymerases or replicases formed in cells infected with RNA viruses (Spiegelman *et al.* 1968; August, 1969; Kit & Dubbs, 1969).

A recent report suggests that exclusively RNA-dependent RNA-synthesizing enzymes may exist in cells not infected by viruses. Neuhoﬀ, Schill & Jacherts (1970) found that competent macrophage cells possess an RNA-dependent RNA polymerase responsible for the synthesis of the specific RNA formed after their contact with an antigen. Since the DNA-dependent polymerase of the extreme halophile *Halobacterium cutirubrum*, unlike those so far isolated from other bacteria, does not accept RNA as primer (Louis & Fitt, 1971), it is notable that this organism also possesses an exclusively RNA-dependent RNA polymerase, which is readily separable from the DNA-dependent enzyme.

This present paper describes the partial purification of the enzyme and some of its properties. A preliminary account of part of this work has been published (Fitt & Louis, 1970).

EXPERIMENTAL

Materials. Yeast tRNA was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and venom phosphodiesterase from Worthington Biochemical Corp., Freehold, N.J., U.S.A. The sources of other materials and intermediates have been described (Louis & Fitt, 1971).

Preparation of *H. cutirubrum* RNA-dependent RNA polymerase. *H. cutirubrum* strain no. 9 cells were grown and harvested as described by Peterkin & Fitt (1971). All subsequent operations were carried out at 0–5°C. Concentrations of $(\text{NH}_4)_2\text{SO}_4$ were calculated by the procedure of Green & Hughes (1955).

Preparation of the crude extract from *H. cutirubrum* cells (24g wet wt.) and the subsequent pH4 precipitation were performed as described for the purification of DNA-dependent RNA polymerase from this organism (Louis & Fitt, 1971). The precipitate from the acid precipitation contained all the RNA-dependent activity, contaminated by a small amount of the DNA-dependent enzyme, and the bulk of the latter remained in the supernatant after centrifugation of the suspension at 27000*g*. The pellet was suspended in the minimum volume of 2.5M-KCl–1M-NaCl–10mM-tris-HCl buffer, pH 8.6 ('high-salt buffer'), and dialysed against high-salt buffer (200 vol.) until it dissolved completely (after 24–36 h). The solution (10–15 ml) was then dialysed for a total of 4 h against two changes (100 vol. each) of 10mM-tris-HCl buffer, pH 8.6. Sufficient $(\text{NH}_4)_2\text{SO}_4$ was added to give a final concentration of 80% of saturation (no allowance being made for any salt still present) and the suspension was stirred for 30 min. The precipitate was removed by centrifugation and the supernatant was dialysed overnight against high-salt buffer. The solution (10–15 ml) was passed through a

column (2.5 cm × 85 cm) of Bio-Gel P-60 and the active fractions were concentrated by dialysis against 50% (w/v) Ficoll in high-salt buffer. Gel filtration was performed in high-salt buffer as described by Louis, Peterkin & Fitt (1971).

Enzyme assays. The RNA-dependent polymerase assay depends on the incorporation of ribonucleoside triphosphates into an acid-insoluble product. The standard assay medium (0.1 ml) contained: tris-HCl buffer, pH 9.5, 10 μmol; one ¹⁴C-labelled nucleoside triphosphate, 5 nmol (about 10000 d.p.m./nmol); the other three nucleoside triphosphates, 20 nmol; high-molecular-weight RNA, 75 μg; MgCl₂, 10 μmol; MnCl₂, 1 μmol; KCl, 150 μmol; NaCl, 60 μmol; enzyme, up to 1.5 units. Incubation was at 37°C for 1 h and samples (up to 85 μl) were then streaked on numbered strips (2 cm × 3 cm) of Whatman no. 3 filter paper. The strips were collected in cold 7% (w/v) HClO₄ (10 ml/strip) and the suspension was swirled for at least 7 min on a rotary shaker. The strips were allowed to settle and the wash fluid was removed with an aspirator. The washing procedure was repeated successively with similar volumes of cold 7% (w/v) HClO₄, 1% (w/v) HClO₄ and ethanol. The washed strips were dried in air and their radioactivities counted to 2% accuracy in a liquid-scintillation counter. Suitable control experiments were performed and their radioactivities subtracted from the assay value. The specific radioactivity of ¹⁴C-labelled nucleoside triphosphate was determined and all radioactivities were converted into d.p.m. as described by Louis & Fitt (1971). One unit was defined as the amount of enzyme catalysing the incorporation of 1 nmol of [¹⁴C]ATP into acid-insoluble material/h in these conditions.

H. cutirubrum DNA-dependent RNA polymerase and deoxyribonuclease and ribonuclease activities were assayed as described by Louis & Fitt (1971).

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

RESULTS AND DISCUSSION

Purification of *H. cutirubrum* RNA-dependent RNA polymerase. The results of a typical purification are shown in Table 1. The bulk of the DNA-dependent polymerase is removed by the acid precipitation and most of the remainder during the next two steps. A large increase in total activity occurs as a result of ammonium sulphate fractionation, presumably owing to removal of degradative

enzymes or other interfering substances, so the overall increase in specific activity of 2800-fold relative to the crude extract gives an artificially high estimate of the relative purification achieved: on the basis of the recovery of protein, a maximum of some 1200-fold would be possible.

The purified enzyme was free from detectable nuclease activity either in the presence or absence of salt. Its approximate molecular weight was determined by gel filtration and sucrose-density-gradient centrifugation (Louis *et al.* 1971) and found to be 17000–18000. This may be compared with molecular weight 150000 reported for the RNA-dependent Q β -replicase (August, 1969).

Enzymic properties of *H. cutirubrum* RNA-dependent RNA polymerase. The results in Tables 2, 3 and 4 show that the enzyme is an RNA-dependent RNA polymerase. The presence of RNA was essential for activity (Table 2) and little or no incorporation took place with either native or denatured calf thymus DNA as template. tRNA was much less effective than high-molecular-weight RNA, and brief pretreatment of the tRNA with venom phosphodiesterase by the method of Preiss, Dieckmann & Berg (1961) decreased its effectiveness instead of increasing it, as would have been expected had the enzyme been an RNA-adenylate (cytidylate) pyrophosphorylase ('CCA-adding enzyme'). The results in Fig. 1 confirm that high-molecular-weight RNA was more effective than tRNA even when a large excess of the latter was used.

All four ribonucleoside triphosphates were required (Table 3) and omission of any one markedly decreased the incorporation of the others. In particular, no detectable incorporation occurred with a mixture of ATP and CTP as substrate, confirming that the activity was not due to a 'CCA-adding enzyme'. Further, in the presence of all four ribonucleoside triphosphates the incorporation in the standard assay was similar to that determined with [¹⁴C]ATP (Table 3, Expt. 1) irrespective of which nucleotide was radioactive.

Table 4 shows that not only was the product of

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

The enzyme was purified as described in the Experimental section. Activity was determined in the standard assay system. A unit is the amount of enzyme catalysing the incorporation of 1 nmol of [¹⁴C]ATP/h. The numbers in parentheses represent the units of DNA-dependent RNA polymerase present in the various fractions.

Fraction	Activity (units)	Protein (mg)	Sp. activity (units/mg)	Relative purification
Crude extract	108 (180)	1080	0.1	1.0
pH 4 precipitate	60 (4.2)	130	0.46	4.6
(NH ₄) ₂ SO ₄ supernatant	345 (2.4)	42	8.3	83
Bio-Gel P-60	252 (0.1)	0.9	280	2800

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

Enzyme activity was determined in the standard assay system with the indicated nucleic acid as template. tRNA was treated with venom phosphodiesterase by the method of Preiss *et al.* (1961). The denatured DNA was prepared by heating a solution of native calf thymus DNA (2mg/ml) at 100°C for 10min and then cooling it in ice.

Template	ATP incorporated (nmol)
None	0
High-molecular-weight RNA (0.75mg/ml)	0.77
tRNA (2.4mg/ml)	0.41
Venom-diesterase-treated tRNA (2.4mg/ml)	0.30
Native DNA (0.3mg/ml)	0.01
Denatured DNA (0.3mg/ml)	0.04

the reaction insensitive to pancreatic deoxyribonuclease, but the latter also failed to stop the reaction, confirming that DNA is not required as template. However, pancreatic ribonuclease hydrolysed the product and decreased incorporation to less than 15% of the expected value. The product is therefore a polynucleotide, and the fact that RNA of high molecular weight is a better primer than three to four times its weight of tRNA (Table 2) suggests that the reaction does not involve terminal addition, since the latter should be favoured by the increased number of end groups in the shorter primer. However, detailed study will be necessary to establish whether new chains are formed and whether the base-sequence of the primer determines that of the product.

The RNA-dependent RNA polymerase has a sharp optimum substrate concentration in the standard assay conditions (Fig. 2), like the DNA-dependent enzyme (Louis & Fitt, 1971). However,

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

Enzymic activity of the purified enzyme was determined by the standard assay with the indicated nucleotides (150nmol/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	0.89
(2) CTP, UTP, GTP	[³ H]CTP	0.05
(3) ATP, UTP, GTP	[¹⁴ C]ATP	0.04
(4) ATP, CTP, GTP	[³ H]CTP	0
(5) ATP, CTP, UTP	[¹⁴ C]ATP	0.10
(6) ATP, CTP	[¹⁴ C]ATP	0
(7) ATP, UTP	[¹⁴ C]ATP	0.03
(8) ATP, GTP	[¹⁴ C]ATP	0.12
(9) CTP, UTP	[³ H]CTP	0
(10) CTP, GTP	[³ H]CTP	0
(11) UTP, GTP	[³ H]UTP	0
(12) ATP	[¹⁴ C]ATP	0.12
(13) CTP	[³ H]CTP	0
(14) UTP	[³ H]UTP	0.06
(15) GTP	[³ H]GTP	0

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

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

Addition after 60 min	[¹⁴ C]ATP incorporated (nmol)
(1) Water, 20 µl (60 min control)	0.93
(2) Water, 20 µl (90 min control)	1.42
(3) Ethanol, 20 µl (reaction stopped)	0.91
(4) Deoxyribonuclease (0.5mg/ml), 20 µl	1.38
(5) Ribonuclease (0.5mg/ml), 20 µl	0.20

Fig. 1. Effect of RNA concentration on activity of *H. cutirubrum* RNA-dependent RNA polymerase. The activity was determined in the standard assay with the indicated concentrations of high-molecular weight RNA (■) or tRNA (●).

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

the optimum concentration for the RNA-dependent activity (200 nmol/ml) is somewhat higher than that for the DNA-dependent polymerase (150 nmol/ml). As in the latter case, it was found that the sensitivity of the standard assay was improved with comparatively little effect on the activity of the enzyme, if labelled nucleoside triphosphate of higher specific radioactivity was used at a lower concentration (50 nmol/ml) in the presence of optimum amounts of the other three non-radioactive nucleoside triphosphates.

Fig. 3. Effect of Mg^{2+} and Mn^{2+} concentrations on the activity of *H. cutirubrum* RNA-dependent RNA polymerase in the standard assay. Each point represents the average of two results differing by less than 10%. ■, $MgCl_2$; ●, $MnCl_2$; □, $MgCl_2 + 10 \mu\text{mol}$ of $MnCl_2/\text{ml}$; ○, $MnCl_2 + 100 \mu\text{mol}$ of $MgCl_2/\text{ml}$.

The enzyme is stimulated by either Mg^{2+} or Mn^{2+} (Fig. 3), although the latter is much more effective. However, when both are present their effects are roughly additive, which suggests that the metal ions act at separate sites or in two independent steps in the overall reaction. These results are quite different from those obtained with *H. cutirubrum* DNA-dependent RNA polymerase (Louis & Fitt, 1971), which has an absolute requirement for both Mg^{2+} and Mn^{2+} and is inactive with either alone.

In the standard assay, incorporation is proportional to the concentration of *H. cutirubrum* RNA-dependent RNA polymerase up to 80 units/ml and linear with time from 0 to 90 min. The enzyme has a sharp pH optimum of 9.5 in the standard conditions and its stability during storage seems constant in the range pH 7.5–10.

The activity of both the DNA-dependent and RNA-dependent enzymes was measured in their respective standard assays in the presence of the other enzyme. The DNA-dependent enzyme was unaffected by the presence of the RNA-dependent polymerase, but the RNA-dependent enzyme was inhibited by the DNA-dependent polymerase, probably as a result of the greater affinity of the latter for nucleoside triphosphates. Since neither enzyme stimulated the other, it seems unlikely that they have a functional relationship *in vivo*, especially in view of the ease with which they can be separated.

Although there has been no report of a bacteriophage that can infect extremely halophilic bacteria, the possibility that the RNA-dependent RNA polymerase is virus-induced must be considered.

Fig. 4. Effect of KCl and NaCl on the activity of *H. cutirubrum* RNA-dependent RNA polymerase. Enzymic activity was determined in the standard assay at the indicated salt concentrations. Each point represents the average of two results differing by less than 10%. ●, KCl; ■, NaCl; ▲, NaCl in the presence of 3M-KCl.

The strain of *H. cutirubrum* used in these studies does not develop plaques on agar nor does it multiply more slowly than other strains of this bacterium, so if a bacteriophage were present it would have to be one that did not interfere with normal cell development. It is therefore extremely improbable that the enzyme is virus-induced, although it will be difficult to exclude this possibility entirely until RNA-viruses infective to extremely halophilic bacteria are discovered and studied. It will also be interesting to establish if other extremely halophilic bacteria also contain RNA-dependent RNA polymerases.

Effect of salts on the stability and activity of H. cutirubrum RNA-dependent RNA polymerase. Enzymes from extremely halophilic bacteria usually require high concentrations of potassium chloride or sodium chloride or of both for both activity and stability. The DNA-dependent RNA polymerase (Louis & Fitt, 1971) and a fatty acyl synthetase (M. Kates, personal communication) from *H. cutirubrum* are unusual in that they are inhibited by salt. The RNA-dependent RNA polymerase is relatively unaffected by salt concentration, but it is still most stable and active in a high-salt environment.

The enzyme is more stable in the presence of salt than in its absence, but the salt requirement is not absolute: when stored at 4°C in 2.5M-potassium chloride-1M-sodium chloride-10mM-tris-HCl buffer, pH 8.6, it lost 27% of its activity in 3 weeks, whereas in salt-free buffer 62% of the activity was lost during the same period.

Fig. 4 shows that the activity of the enzyme increases slightly with potassium chloride or sodium chloride concentration up to about 3M. It is most active in a mixture of the two salts with an optimum in 3M-potassium chloride-1M-sodium chloride, but nevertheless it still retains half its maximum activity in the absence of salt.

Thus the RNA-dependent RNA polymerase differs markedly from the DNA-dependent enzyme so far as its salt requirements are concerned. For an enzyme from an extreme halophile it is fairly stable in the absence of salt, whereas the DNA-dependent RNA polymerase is very labile in a low-salt environment. However, although the DNA-dependent polymerase is strongly inhibited by univalent cations, the RNA-dependent enzyme is stimulated by high concentrations of potassium chloride and sodium chloride similar to those found in living *H. cutirubrum* cells. The activities of the two enzymes in crude extracts of the bacteria are therefore about the same when measured at high-salt concentrations so it is possible that RNA-dependent RNA polymerase plays some important role in RNA synthesis in extremely halophilic bacteria.

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