Nucleic Acid Enzymology of Extremely Halophilic Bacteria

GEL-FILTRATION AND DENSITY-GRADIENT-CENTRIFUGATION STUDIES OF THE MOLECULAR WEIGHTS OF *HALOBACTERIUM CUTIRUBRUM* POLYNUCLEOTIDE PHOSPHORYLASE AND DEOXYRIBONUCLEIC ACID- AND RIBONUCLEIC ACID-DEPENDENT RIBONUCLEIC ACID POLYMERASES

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1. Conditions have been established for the estimation of molecular weights of proteins by analytical gel filtration and sucrose-density-gradient centrifugation in 2.5M-potassium chloride-1M-sodium chloride; *Halobacterium cutirubrum* polynucleotide phosphorylase, DNA-dependent RNA polymerase and RNA-dependent RNA polymerase have been studied by these methods. 2. The RNA-dependent polymerase has also been studied by density-gradient centrifugation in the absence of salt. 3. All three proteins are of unusually low molecular weight compared with similar enzymes from non-halophilic bacteria.

During the isolation and partial purification from *Halobacterium cutirubrum* of its polynucleotide phosphorylase (nucleoside diphosphate-polynucleotide nucleotidyltransferase; EC 2.7.7.8) (Peterkin & Fitt, 1970, 1971), DNA-dependent RNA polymerase [nucleoside triphosphate-RNA nucleotidyltransferase (DNA-dependent); EC 2.7.7.6] (Fitt & Louis, 1970; Louis & Fitt, 1971a) and RNA-dependent RNA polymerase (Fitt & Louis, 1970; Louis & Fitt, 1971b) it was observed that all three enzymes were highly retarded during gel filtration. It seemed probable that they had unusually low molecular weights compared with those of the corresponding enzymes from non-halophilic bacteria, in agreement with the prediction made by Ingram (1947) that enzymes from extreme halophiles might be smaller than their non-halophilic counterparts.

A systematic study of the molecular weights of all three enzymes was therefore undertaken by gel filtration and sucrose-density-gradient centrifugation, and the results of these experiments are reported in the present paper.

EXPERIMENTAL

Materials. Gel-filtration materials and protein molecular-weight standards were purchased from the following suppliers: Bio-Gel P-60 polyacrylamide gel, Bio-Rad Laboratories, Richmond, Calif., U.S.A.; Sephadex G-50, Sephadex G-200 and Blue Dextran, Pharmacia (Canada) Ltd., Montreal, P.Q., Canada; bacitracin and sperm-whale myoglobin, Mann Research Laboratories, Orangeburg, N.Y., U.S.A.; crystalline bovine serum albumin,

ovalbumin grade V, α -chymotrypsinogen A type II (ox pancreas) and cytochrome *c* type VI (horse heart), Sigma Chemical Co., St Louis, Mo., U.S.A.

Highly-purified *Azotobacter vinelandii* polynucleotide phosphorylase (Gajda, Zaror de Behrens & Fitt, 1970) was kindly provided by Mr A. T. Gajda; *H. cutirubrum* polynucleotide phosphorylase (Sephadex G-200, peak II fraction), was purified by the method of Peterkin & Fitt (1971); *H. cutirubrum* DNA-dependent and RNA-dependent RNA polymerases were purified as described by Louis & Fitt (1971a,b).

Enzyme assays. The polynucleotide phosphorylases were assayed by their respective standard phosphorolysis assays (Gajda *et al.* 1970; Peterkin & Fitt, 1971) and the two RNA polymerases by their standard assays (Louis & Fitt, 1971a,b).

Gel-filtration chromatography. Sephadex K25/45 and K25/100 columns equipped with flow adapters were used in all the gel-filtration experiments.

All the gels were allowed to swell in 2.5M-KCl-1M-NaCl-10mM-tris-HCl buffer, pH 8.6 ('high-salt buffer'), for at least 4 weeks at 4°C before use. The high-salt buffer contained 0.02% (w/v) NaN₃ to prevent microbial growth. Sephadex G-200 swollen for less than 4 weeks in these conditions gave unstable columns and irreproducible results, since the dry gel regains water very slowly in buffers of high ionic strength (Peterkin & Fitt, 1971). A detailed study of the rate of swelling of the other gels was not made, so it was considered safer to prepare them by the method used for Sephadex G-200, although they did appear to swell faster than the latter. Fines were removed by repeated decantation and resuspension in high-salt buffer.

The column fitted with its lower flow adapter, was cooled to 2-5°C in a cold-room, mounted vertically (by spirit level) and fitted with a Sephadex R-25 column extender. Enough high-salt buffer to fill the column to a height of

about 8 cm above the lower adapter was added and air bubbles were carefully removed from the latter with the aid of a long glass tube. Sufficient gel slurry for the complete bed was added all at once and the gel allowed to settle under gravity for 20 min before the flow of buffer was started. For Sephadex G-200 and Bio-Gel P-60, the downward flow of buffer was then started and maintained for 12–18 h by a maximum hydrostatic pressure of 10 cm. The upper flow adapter was installed and a further column volume of high-salt buffer run downwards through the bed. The flow of buffer was then reversed and maintained at 10 ml/h (Sephadex G-200) or 15 ml/h (Bio-Gel P-60) for the duration of the experiment by means of a peristaltic pump under a hydrostatic pressure of 2–5 cm. The harder Sephadex G-50 was packed under a hydrostatic pressure of 1–2 m and 2 column volumes of high-salt buffer were passed through the bed before installation of the upper flow adapter. The flow was reversed, the hydrostatic pressure lowered to 0.5 m and a further 1 column volume of high-salt buffer passed through the bed. The flow-rate was then decreased and maintained at 20 ml/h for the duration of the experiment by means of a peristaltic pump.

Samples were applied via a three-way valve to avoid interrupting the flow of buffer. Each sample was 'chased' on to the column with 2 ml of 10% (w/v) sucrose in high-salt buffer. The E_{280} of the effluent was monitored continuously (LKB Uvicord II) and 4 ml fractions were collected. The extinctions of the fractions were accurately determined with a Zeiss PMQ II spectrophotometer at (i) 405 nm for cytochrome *c*, (ii) 280 nm for other proteins and Blue Dextran and (iii) 257 nm for adenosine. The elution volume (V_e) of a substance was determined at the maximum height of the corresponding peak (Fischer, 1969).

The void volume (V_0) and adenosine elution volume were measured simultaneously. A 2 ml sample consisting of 0.2% (w/v) Blue Dextran and about 10 μ mol of adenosine/ml in high-salt buffer was centrifuged briefly at 27 000g to remove undissolved material and then passed through the column at the start of each experiment, immediately after the packing and stabilization of the bed. Blue Dextran is excluded from the gels and therefore emerges with the void volume. The adenosine elution volume was assumed to be equal to the sum of V_0 and the internal volume (V_i). The bed volume (V_t) was calculated from the length of the gel bed and the internal diameter of the column (2.5 cm).

Standard proteins were dissolved in high-salt buffer and turbid solutions were cleared by a brief centrifugation at 27 000g. The standards were applied individually to the columns in samples containing 7–10 mg in 0.5–1.5 ml. The size and concentration of samples of enzymes (each applied individually) were determined by their availability: amounts adequate for detection by the standard assays were necessarily used, but larger samples were loaded where possible to permit continuous monitoring of the E_{280} of the effluent and thus facilitate detection of the peaks of activity.

For each standard, the average partition coefficient K_{av} was calculated from the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$ (Fischer, 1969). These values were plotted against the logarithm of the corresponding molecular weight (Detemmann, 1967; Fischer, 1969) and a straight line was fitted to the linear portion of the curve by the method of least squares. The molecular weights of the

enzymes were then calculated from their K_{av} values by using the fitted equation.

Density-gradient centrifugation. Determination of molecular weights by sucrose-density-gradient centrifugation was carried out by a modification of the method of Martin & Ames (1961).

Linear sucrose gradients (5–20%, w/v) in 10 mM-tris-HCl buffer, pH 8.6, or high-salt buffer were prepared by using a Buchler gradient former. It should be noted that the gradients prepared in high-salt buffer had an average homogeneous density of 1.187 g/ml, which approaches the safe limit for many rotors, so these salt concentrations should not be exceeded.

For polynucleotide phosphorylase, 4.8 ml gradients were formed in Beckman Polyallomer tubes 1.3 cm in diam. and 5.1 cm long. The other experiments were performed with 4.3 ml gradients in 1.1 cm $\times$ 6.0 cm Beckman Polyallomer tubes. Samples (0.2 ml) were layered on top of each gradient. The sample concentration was 7–10 mg/ml for the standard proteins; enzyme concentration was maintained well above the limit of detectability in the standard assay system.

The experiment with polynucleotide phosphorylase was run at 280 000g for 24 h in the SW-65L rotor of a Beckman L2-65B preparative ultracentrifuge. The other experiments were performed with the SW-56 rotor at 308 000g for 15.5 h. All runs were carried out at 5–7°C (lower temperatures led to the co-precipitation of salt and protein in the high-salt gradients). The tubes were pierced and 30–40 fractions/tube (about 140 μ l/fraction) were collected. Protein concentration was determined by diluting 0.05 ml samples of each fraction to 1 ml with water and measuring the E_{280} (E_{405} for cytochrome *c*) in a Zeiss PMQ II spectrophotometer. The distance of the fraction containing the highest concentration of a substance from the rotor centre at the end of the run, referred to as r_s , was calculated for each standard and each enzyme.

To facilitate calculation of the molecular weight a constant (k) was defined as being that fraction of the gradient through which the substance (*s*) moves during a run, i.e. $k = (r_s - r_{min.})/(r_{max.} - r_{min.})$ where $r_{max.}$ is the radius to the bottom of the gradient and $r_{min.}$ that to the top. The k values thus calculated were plotted against the logarithm of the molecular weight and the enzyme molecular weights estimated as described above for gel-filtration experiments. [Similar results were obtained by plotting $\log k$ against $\log$ (mol.wt.).]

RESULTS

Comparison of the properties of dextran and polyacrylamide gels swollen in water or high-ionic-strength buffer. The void and internal volumes of columns of Sephadex G-200, Sephadex G-50 and Bio-Gel P-60 prepared and run in 2.5 M-potassium chloride–1 M-sodium chloride–10 mM-tris-HCl buffer, pH 8.6 (high-salt buffer), are compared in Table 1 with the calculated values (based on the manufacturers' specifications) for similar columns of these gels prepared in water. The results obtained for Sephadex G-50 and Bio-Gel P-60 swollen in high-salt buffer do not differ significantly from the predicted values for the same gels swollen in water.

However, the properties of Sephadex G-200 columns are greatly influenced by the ionic strength of the liquid medium: high salt concentrations seem to lower the total water regain of the gel, as well as decreasing its rate of swelling, so there is less space within the gel (V_i) and correspondingly more space between gel particles (V_0). The swollen gel no longer occupies 30–40 ml/g dry wt. of gel (Determann, 1967), and 30 g (dry wt.) of Sephadex G-200 swollen in high-salt buffers is required to pack a 430 ml column.

The changes in the column parameters discussed above profoundly influence the relationship between the molecular weight of a substance and its elution volume during Sephadex G-200 gel filtration. For a column of gel swollen and used in water this relationship is given by the equation (Determann, 1967):

$$\log(\text{mol. wt.}) = 6.698 - 0.987(V_e/V_0)$$

whereas the corresponding equation for the gel swollen in high-salt buffer is:

$$\log(\text{mol. wt.}) = 7.306 - 1.6462(V_e/V_0).$$

Fig. 1 shows that in high-salt buffer Sephadex G-200 may be used to determine molecular weights

in the range 12500–65000, as opposed to 12500–200000 in the normal buffers of low ionic strength (Determann, 1967). It is probable that the lower limit is also decreased in high-salt conditions, but we have not yet been able to check this point owing to a lack of suitable standard proteins. Precise definition of the upper limit will also require the running of additional standards. However, within the indicated range there is a linear relationship between elution volumes and molecular weights of the standard proteins used.

Fig. 1. Sephadex G-200 gel filtration. The column was packed and run in high-salt buffer as described in the Experimental section. ●, Cytochrome *c*; ■, α -chymotrypsinogen A; ▲, ovalbumin; ○, bovine serum albumin; △, *A. vinelandii* polynucleotide phosphorylase; □, *H. cutirubrum* polynucleotide phosphorylase.

Table 1. Comparison of the column parameters of Sephadex G-200 and G-50 and of Bio-Gel P-60 in the presence and absence of salt

The available manufacturers' specifications were used to calculate the void and internal volumes of hypothetical salt-free columns whose bed volumes were equal to the measured bed volumes of our high-salt columns. The void and internal volumes of the latter were measured as described in the Experimental section.

Gel	V_t	Without salt		With salt	
		V_0	V_i	V_0	V_i
G-200	431	129	287	172	258
G-50	179	71.6	89.5	71.3	93.0
P-60	435	165	—	159	191

Table 2. Standard proteins used in determinations of molecular weight

The proteins shown were used as molecular-weight standards in the experiments described

Protein	Molecular weight	Reference
(1) Bacitracin	1450	Schröder & Lübke (1966)
(2) Cytochrome <i>c</i> (horse heart)	12400	Fish, Mann & Tanford (1969)
(3) Myoglobin (sperm whale)	17800	Determann (1967)
(4) α -Chymotrypsinogen A (bovine pancreas)	25000	Determann (1967)
(5) Ovalbumin	45000	Determann (1967)
(6) Bovine serum albumin	67000	Determann (1967)
(7) Polynucleotide phosphorylase (<i>A. vinelandii</i>)	200000	Thang (1969)

Fig. 2. Sephadex G-50 gel filtration. The column was packed and run in high salt buffer as described in the Experimental section. ●, Cytochrome *c*; ▲, myoglobin and *H. cutirubrum* DNA-dependent RNA polymerase, which had the same K_{av} ; ■, α -chymotrypsinogen A; △, *H. cutirubrum* RNA-dependent RNA polymerase; □, *H. cutirubrum* polynucleotide phosphorylase.

Fig. 3. Sephadex G-50 gel filtration with scale extended to include bacitracin (○, other symbols are defined in the legend to Fig. 2). The error of extrapolating the straight line to include polynucleotide phosphorylase is clearly small.

A list of the molecular-weight standards used in our studies with references to the literature source of their molecular weights, is given in Table 2.

Molecular weight of H. cutirubrum polynucleotide

Fig. 4. Sucrose-density-gradient centrifugation in high-salt buffer of *H. cutirubrum* polynucleotide phosphorylase. The centrifugation was carried out as described in the Experimental section. ●, Cytochrome *c* and the enzyme, which penetrated the gradient to the same extent; ▲, myoglobin; ■, α -chymotrypsinogen A. Each sample was run in a separate tube.

phosphorylase. H. cutirubrum polynucleotide phosphorylase was chromatographed on standardized Sephadex G-200 (Fig. 1) and Sephadex G-50 (Figs. 2 and 3) columns, and was examined by sucrose-density-gradient centrifugation in high-salt buffer (Fig. 4). The enzyme was eluted from both gel columns after the smallest standard protein, cytochrome *c*, but the position of bacitracin (mol.wt. 1450) on the Sephadex G-50 curve (Fig. 3) suggests that the error of extrapolating the straight line is small, thus permitting an estimate of the molecular weight to be made. In the sucrose gradient, the enzyme was found in the same position as cytochrome *c*. The estimates of molecular weight by the three methods were: (i) <12400 (Sephadex G-200); (ii) 11200 (Sephadex G-50); (iii) 12400 (gradient centrifugation). It is therefore probable that the molecular weight of the enzyme is in the range 11000–12000.

Molecular weight of H. cutirubrum DNA-dependent RNA polymerase. This enzyme was chromatographed in high-salt buffer on Sephadex G-50 (Fig. 2) and Bio-Gel P-60 (Fig. 5); and centrifuged in a sucrose density gradient in high-salt buffer

Fig. 5. Bio-Gel P-60 gel filtration. The column was packed and run in high-salt buffer as described in the Experimental section. ●, Cytochrome *c*; ■, α -chymotrypsinogen A; ▲, ovalbumin; ○, *H. cutirubrum* DNA- and RNA-dependent RNA polymerases, which had the same K_{av} .

Fig. 6. Sucrose-density-gradient centrifugation, in high-salt buffer, of *H. cutirubrum* DNA- and RNA-dependent RNA polymerases. The centrifugation was carried out as described in the Experimental section. ●, Cytochrome *c*; ■, α -chymotrypsinogen A; ▲, ovalbumin; △, *H. cutirubrum* RNA-dependent RNA polymerase; ○, *H. cutirubrum* DNA-dependent RNA polymerase. Each sample was run in a separate tube.

Fig. 7. Sucrose-density-gradient centrifugation, in the absence of salt, of *H. cutirubrum* RNA-dependent RNA polymerase. The centrifugation was carried out as described in the Experimental section. Symbols are defined in the legend to Fig. 6. Each sample was run in a separate tube.

(Fig. 6): the resulting estimates of molecular weight were 17 900, 17 800 and 20 900 respectively. Owing to the greater precision possible in gel-filtration experiments, the molecular weight is probably close to 18 000.

Molecular weights of *H. cutirubrum* RNA-dependent RNA polymerase. The molecular weight of this enzyme was estimated as described above by gel filtration in high-salt buffer on Sephadex G-50 (Fig. 2) and Bio-Gel P-60 (Fig. 5) and by high-salt sucrose-density-gradient centrifugation (Fig. 6). However, in this case the relatively high stability of the RNA-dependent polymerase at low-salt concentrations (Louis & Fitt, 1971*b*) allowed a further estimate of the molecular weight to be made by sucrose-density-gradient centrifugation of the enzyme and standards in the absence of salt (Fig. 7) by the classical procedure of Martin & Ames (1961). The four values obtained for the molecular weight of the RNA-dependent polymerase were 16 800, 17 800, 18 200 and 16 200 with an average of 17 400. As expected, the results obtained by gel filtration differ less than those from the sucrose-density-gradient centrifugations.

DISCUSSION

The molecular weights estimated for the three *H. cutirubrum* enzymes from the experiments described above are compared in Table 3 with those

Table 3. *Comparison of the molecular weights of the enzymes from H. cutirubrum with those of enzymes from non-halophilic bacteria*

Enzyme	Source	Molecular weight	Reference
(1) Polynucleotide phosphorylase	<i>A. vinelandii</i>	200 000	Thang (1969)
	<i>H. cutirubrum</i>	11 000–12 000	
(2) DNA-dependent RNA polymerase	<i>E. coli</i>	400 000	Burgess (1969)
	<i>H. cutirubrum</i>	18 000	
(3) Q β -replicase	Q β -infected <i>E. coli</i>	150 000	August (1969)
	<i>H. cutirubrum</i>	17 000–18 000	
RNA polymerase			

of corresponding or similar enzymes from non-halophilic bacteria. The *H. cutirubrum* enzymes all appear much smaller than their non-halophile counterparts and the molecular weight of the polynucleotide phosphorylase suggests that it is among the smallest enzymes known. It is therefore essential to exclude the possibility that some abnormal properties of the *H. cutirubrum* enzymes themselves, or alternatively some anomalous behaviour of the standard proteins in a medium of high ionic strength, could be responsible for the results.

There are three possible sources of error that might account for the gel-filtration results. First, the *H. cutirubrum* enzymes might adopt abnormally compact configurations in the presence of high concentrations of salt and thus be eluted later than would be expected on the basis of their true molecular weights. Second, the *H. cutirubrum* enzymes might be selectively adsorbed on the gels owing to some unknown structural features and once again be eluted abnormally late. Third, the non-halophilic protein standards might associate in the presence of salt and thus be eluted sooner than expected.

The first possibility is eliminated by the results of the sucrose-density-gradient centrifugation experiments in high-salt buffer. If the *H. cutirubrum* enzymes were abnormally compact, and therefore dense, in conditions of high ionic strength they should sediment rapidly and would appear to have unusually high molecular weights. The results of the gradient-centrifugation and gel-filtration experiments should therefore differ markedly whereas in fact they agree remarkably well. Thus *H. cutirubrum* enzymes cannot be abnormally compact.

It is known (Janson, 1967) that molecules with aromatic or conjugated structures can be adsorbed to Sephadex gels. These effects are usually more marked with the more highly cross-linked gels such as Sephadex G-25 and of little or no importance with Sephadex G-200. In any case, the possibility that the *H. cutirubrum* enzymes are selectively adsorbed to any of the three gels used in our work is also

eliminated by the sucrose-density-gradient-centrifugation results, since once again this method should give a significantly higher estimate of molecular weight than gel filtration if the proteins were adsorbed to the gels.

Finally, the possibility that some abnormal behaviour of the standards could occur in the high-salt buffer is eliminated by the result of the experiments with sucrose-density-gradient centrifugation in standard classical conditions of low ionic strength. *H. cutirubrum* RNA-dependent RNA polymerase is comparatively stable in the absence of salt and the value for its molecular weight determined in these conditions was similar to that obtained by the gel-filtration and gradient-centrifugation experiments in the high-salt buffer.

Thus there seems no reason to doubt the validity of molecular-weight estimation by gel filtration and density-gradient centrifugation in these conditions, or that the molecular weights observed are in the correct range for the molecules we have studied. However, it might be argued that in the conditions of the assays for enzymic activity the three enzymes could associate to give the true functional units. It is difficult to eliminate such a possibility, but there also appears to be little precedent for it. *Escherichia coli* RNA polymerase associates to give larger active complexes, but the smallest homogeneous particle is the functional entity (Seifert & Zillig, 1969), which on further dissociation yields several distinct, inactive species.

The results of our experiments thus support Ingram's (1947) prediction that the reason for the solubility of halophile enzymes at the high intracellular salt concentration would prove to be their involvement 'in smaller molecular aggregates'. However, it is clear that this is not true of all enzymes from extremely halophilic bacteria, since *H. cutirubrum* catalase is a large molecule (Lanyi & Stevenson, 1969) and we have observed proteins with elution volumes consistent with molecular weights up to 200 000 during the gel-filtration steps in the purification of the three enzymes we have studied.

It will clearly be of great interest to extend these studies to other enzymes of nucleic acid metabolism in extreme halophiles to determine whether they constitute a group of abnormally small proteins compared with other enzymes from these bacteria. Further, the three enzymes discussed in this paper appear small enough to permit easy determination of their amino acid sequences if they can be obtained sufficiently pure.

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