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METAL CONTENT OF DNA POLYMERASE I PURIFIED FROM OVERPRODUCING AND WILD TYPE ESCHERICHIA COLI

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DNA polymerase I purified from both E. coli strain B, and from an overproducing E. coli stain lysogenized with a λ pol A phage were analyzed for metal content. After gel filtration to remove loosely bound metals, DNA polymerase I from both strains contained ≤ 0.2 gm atoms Zn^{2+}/mole enzyme and 0.09 to 0.7 Mg^{2+}/mole enzyme. Substoichiometric amounts of Fe, Co, Ni (≤0.2 gm atoms), and Mn (≤ 0.1 gm atoms) were detected. Since the metal content does not correlate with enzymatic activity, we conclude that DNA polymerase I is not a metalloenzyme.

It is well known that E. coli DNA polymerase I requires an added divalent cation, either Mg^{2+} or Mn^{2+}, for activity in vitro. In addition, early investigations suggested that a tightly bound Zn^{2+} was also required for polymerase activity (1,2). Recently, fully active pol I[1] purified from an E. coli strain lysogenized with a λ pol A phage was shown to contain only 0.13 g atom of Zn^{2+} per mole of enzyme (3). We have confirmed this observation and extended it to other metals and to DNA polymerase I from wild type E. coli. Reactivation of the enzyme by contaminating Zn^{2+} in the assay was ruled out by using enzyme concentrations in excess of the Zn^{2+} concentration.

Materials and Methods

E. coli strain (CM5199) lysogenized with λ pol A phage (4) was generously provided by Dr. William E. Brown of Carnegie-Mellon University, Pittsburgh, PA. Tris (Trisma base), thymine, dithiothreitol, mercaptoethanol (Sigma), [^{3}H]dATP (Amersham), Poly d(A-T), dATP, TTP (P-L

[1] Abbreviations used: pol I, DNA polymerase I from Escherichia coli; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate

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Biochemicals), ultrapure ammonium sulfate (Schwartz/Mann), polyethylenimine (Bethesda Research Laboratories), ultrapure $MgCl_2$ (Accurate Chemical and Scientific Corp.), CDTA (Aldrich), and ultrapure $ZnCl_2$ (Alfa Products) were purchased in the highest grade commercially available. Phosphocellulose (P 11) and DEAE cellulose (DE 23) were purchased from Whatman. Sephadex G-100 and G-25 were purchased from Sigma. Hydroxylapatite (Bio Gel HTP) and Chelex-100 were purchased from Bio-Rad Laboratories.

Protein concentrations of pol I were measured spectrophotometrically using a molar extinction coefficient at 280 nm of $9.26 \times 10^4 M^{-1} cm^{-1}$ (5). SDS polyacrylamide slab gel electrophoresis using 7.5% gels was performed according to the method of Laemmli (6). The gels were scanned with a Hoefer Scientific Instruments GS300 transmittance scanning densitometer. Enzyme activity was assayed according to the method of Setlow (7) using poly d(A-T) as primer template, and TTP and [^{3}H]dATP as substrates. In assays where minimal Zn^{2+} contamination was essential, 67 mM Tris Cl, pH 7.5, and 100 mM KCl replaced the 67 mM K+ phosphate buffer used in the final assay solutions and all components except the $MgCl_2$ were made "metal-free" by passing down a Chelex-100 column. The $MgCl_2$ present in the assay contributed less than 0.04 μM Zn. All enzyme dilutions were performed immediately prior to starting the assays.

Two preparations of pol I from the lysogenized E. coli strain (CM5199), designated as preparations 1 and 2, were initially purified by the method of Kelley and Stump (8). Additional purification was found to be necessary (3). Preparation 1 was treated by passage down a 2.5 x 90 cm Sephadex G-100 column equilibrated in 0.1M potassium phosphate, pH 6.5, 0.1M ammonium sulfate, and 1.0 mM mercaptoethanol. The fractions containing pol I were pooled and the phosphocellulose column step was repeated, yielding 80% pure enzyme by gel electrophoresis. Preparation 2 was purified by additional phosphocellulose and hydroxylapatite columns to 91% purity as judged by gel electrophoresis. Two preparations of pol I purified from E. coli strain B, designated as preparations 3 and 4, were utilized. Although these enzymes were several years old, they had been stored at -70°C or lower and their metal content should not have changed. Preparation 3 had been purified as previously described (1) to 99% homogeneity, but at the time of metal analysis had degraded by 20% as judged by gel electrophoresis. Preparation 4, provided by Dr. Paul T. Englund of Johns Hopkins Medical School, corresponds to fraction VI (5), and at the time of analysis, it was 60% pure by gel electrophoresis. E. coli RNA polymerase, purified by the method of Burgess and Jendrisak (10), was reported to be approximately 96% pure by gel electrophoresis (11).

To remove loosely bound contaminating metals ions, an aliquot of each purified enzyme was applied to a Sephadex G-25 column, which had been pre-washed with sodium EDTA (10 mM, pH 7.5) and equilibrated with Chelex-100-treated Tris Cl buffer (50 mM, pH 7.5). Generally 1-2 ml of enzyme in storage buffer containing K^+ phosphate was applied to a 1.5 x 25 cm column. Enzyme treated in this manner contained undetectable phosphate (<1 mM) as determined by the method of Chen (9). We have generally found phosphate buffers to be contaminated with Zn^{2+} which is not completely removed by treatment with Chelex-100. Metal analyses were performed using a Perkin Elmer 370 atomic absorption spectrometer as previously described (1). In one preparation of enzyme, Mn^{2+} content was analyzed by electron paramagnetic resonance spectroscopy of a perchloric acid extract (12).

Results and Discussion

Metal Analyses and Activity Assays

As shown in Table I, specific activities of pol I generally increased slightly after passage down the "metal-free" Sephadex G-25 column, while in

several cases, the metal content decreased substantially. Enzyme contaminated with loosely bound cations might be expected to behave in such a manner. As previously reported (3), pol I from the lysogenized E. coli strain (CM5199) contained very little Zn^{2+} as purified, even without gel filtration to remove loosely bound Zn^{2+}. Wild type pol I, however, contains variable amounts of contaminating Zn^{2+} which might explain the earlier findings of Zn^{2+} in DNA polymerase I (1,2). This Zn^{2+} is effectively removed by the added "metal free" gel filtration step. Of the other metal ions surveyed (Table I), only Mg^{2+} was found in significant quantities in all preparations, while Fe, Co, Ni, and Mn were undetected. The Mg^{2+} content, however, was reduced to substoichiometric levels by gel filtration under "metal free" conditions, and no correlation was found with enzymatic activity. As a test of the methods used, purified E. coli RNA polymerase was found to contain 2 Zn^{2+}/mole before and after gel filtration, while the Mg^{2+} decreased to substoichiometric levels (Table I), as previously reported (13).

Effects of Altering Zn^{2+} and Mg^{2+} Concentrations

Since pol I is generally assayed at $<10^{-9}$M enzyme (7), even undetectably low Zn^{2+} contamination in the assay ($<10^{-7}$M) might reactivate an apoenzyme. To test this possibility, assays were performed at 2°C to slow the reaction, and at concentrations of pol I exceeding the measured Zn^{2+} concentration in the assay (Table II). Lowering the temperature greatly decreased the specific activity of pol I. Raising the Zn^{2+} content from 0.27 Zn^{2+}/enzyme to 2.0 Zn^{2+}/enzyme did not increase, but slightly inhibited the activity.

Since tightly bound Mg^{2+} was found in all pol I preparations, an attempt was made to clarify its role. Preincubation of preparation 2 (1.5 mg/ml) at 4°C for 1 day with $MgCl_2$ (0.1 to 5.0 mM) had negligible effects on specific activity. In another series of experiments, prolonged preincubation with 1.0 to 10 mM of the potent Mg^{2+} chelator CDTA (14) followed by $>10^3$-fold dilution of the enzyme into the assay which contained 6.7 mM excess $MgCl_2$ had negligible effects on activity.

Table I. Metal Analysis and Activity of E. coli DNA Polymerase I and E. coli RNA Polymerase

Enzyme	Source	Specific Activity When Purified (units/mg)	Specific Activity When Analyzed (units/mg) A	Specific Activity When Analyzed (units/mg) B	Metal Content (gm atoms/mole enzyme) Zn A	Zn B	Mg A	Mg B	Fe B	Co B	Ni B	Mn B
Pol I Preparation 1	lysogenized E. coli (CM5199)	11,400	11,400	9,650	≤0.1	≤0.1	3.4	0.70	≤0.2	≤ 0.2	≤0.2	≤0.1[a]
Pol I Preparation 2	lysogenized E. coli (CM5199)	6,160	6,160	7,350	0.10	0.15	0.87	0.31	≤0.2	≤0.2		
Pol I Preparation 3	E. coli B	24,000[b]	2,550	2,890	0.65	0.21		0.40	≤0.2			
Pol I Preparation 4	E. coli B	10,000	1,370	2,390	0.09	0.06	0.28	0.09	≤0.2	≤0.2	≤0.2	≤0.1
RNA Polymerase	E. coli K12	400			1.80	1.92	1.49	0.41				

Columns labeled "A" refer to enzyme before passage through a "metal-free" Sephadex G-25 column (see Methods section), while columns labeled "B" refer to enzyme after passage down the column.

[a]Determined by electron paramagnetic resonance spectroscopy of a 7% W/V perchloric acid extract of DNA polymerase I.

[b]Assay as described in (1), using activated calf thymus DNA.

Table II. Effect of Zn^{++} on Reaction Rate

[Pol I] (μM)	[Zn^{++}] (μM)	Specific Activity (units/mg)
1.94	0.53	27
1.94	3.86	17

Pol I (preparation 2) was passed down the "metal-free" Sephadex G-25 column. One aliquot of this enzyme in 50 mM Tris Cl buffer, pH 7.5, was made 20 μM in Zn^{2+} using a 420 μM $ZnCl_2$ solution. This same volume of deionized distilled H_2O was added to another aliquot. The protein concentration was 11.6 μM, and the aliquots were incubated at 2°C for 10 minutes. Assay solutions were preincubated at 2°C for 15 minutes prior to starting the reaction with either of these two enzyme solutions. The assay mixture (see Methods section) contained 33 μM [^{3}H]dATP, 33 μM TTP, 27 μg/ml poly d(A-T), 100 mM KCl, 67 mM Tris Cl buffer, pH 7.5, 1 mM mercaptoethanol, and 6.67 mM $MgCl_2$. Reactions were stopped after 2 minutes at 2°C.

Discussion

In extending the work of Walton et al (3), we find the metal contents of pol I from both lysogenized and wild type E. coli to be very similar after "metal free" gel filtration (Table I). Neither appears to be a metalloenzyme and replacement of Zn^{2+} by another cation is unlikely. Also, the possibility that Zn^{2+} contamination in the assay might be reactivating the enzyme (3) is unlikely (Table II).

Initial reports suggesting pol I to be a Zn^{2+}-metalloenzyme (1,2) were based on the presence of stoichiometric Zn^{2+} in the enzyme after dialysis, a rapid, reversible inhibition by 1,10-phenanthroline, a slow inactivation and a parallel loss of Zn^{2+} upon dialysis with 1,10-phenanthroline, and slow reactivation by Zn^{2+}, Mn^{2+}, or Co^{2+}. Possible explanations of these findings are that the tightly bound Zn^{2+} contaminant was not removed by simple dialysis. Pol I is known to bind divalent cations tightly at the active site (12). The rapid inhibition of pol I was probably caused by the Cu^{+} complex of 1,10-phenanthroline which cleaves DNA into inhibitory fragments (15). The slow inactivation resulted from either denaturation (3), or an effect of 1,10-phenanthroline not dependent on its chelating ability, and reactivation by cations might have resulted from the removal of

inhibitory 1,10-phenanthroline by metals. The latter reactivation has not been a consistent finding in our hands.

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