Saccharomyces cerevisiae DNA-Dependent RNA Polymerase III: A Zinc Metalloenzyme†

Theodore M. Wandzilak and Robert W. Benson*

ABSTRACT: Yeast nuclear RNA polymerase III was purified by batch adsorption to phosphocellulose, followed by ion-exchange chromatography on DEAE-Sephadex and affinity chromatography on DNA-Sepharose. Polyacrylamide gel electrophoresis of the purified enzyme showed a single protein band which contained polymerase activity. The molecular weight estimated by sedimentation velocity centrifugation in a glycerol gradient was 380 000. Enzyme activity was inhibited 50% at 0.1 mM 1,10-phenanthroline and 100% at 1.0 mM, but was restored when 1,10-phenanthroline was removed by dialysis. Enzyme activity was not inhibited by 7,8-benzoquinoline, a nonchelating structural analogue of 1,10-phenanthroline. These results strongly suggest that inhibition of enzyme activity occurs by the formation of a reversible enzyme–zinc–phenanthroline ternary complex. The zinc content, measured by atomic absorption spectroscopy, was 2 g-atoms per mol of enzyme. Zinc was not removed from the enzyme by gel filtration on Sephadex G-25, by passage through Chelex-100 resin, or by dialysis against buffer containing 1,10-phenanthroline. Enzyme-bound zinc was removed by dialysis after denaturation of the enzyme with heat and sodium dodecyl sulfate. Enzyme-bound zinc did not exchange with free zinc. These results establish yeast nuclear RNA polymerase III as a zinc metalloenzyme.

In 1934 zinc was shown to be essential for normal growth and development of rodents (Todd et al., 1934). Zinc is now known to be an essential nutrient for all phyla. In all species examined thus far, zinc deficiency results in either severe or complete retardation of growth (Vallee, 1959). The profound metabolic disturbances resulting from zinc deficiency point to events critical to cell division and nucleic acid metabolism. Direct evidence that zinc plays an essential role in replication and transcription comes from the demonstration that a number of DNA-dependent DNA polymerases and DNA-dependent RNA polymerases are zinc metalloenzymes (Vallee, 1976). Several RNA-dependent DNA polymerases are also zinc metalloenzymes (Auld et al., 1974, 1975; Poiesz et al., 1974).

Atomic absorption spectroscopy was used to demonstrate the presence of 1 g-atom of zinc per mol of *E. coli* DNA polymerase (Slater et al., 1971). The chelating agent 1,10-phenanthroline inhibited enzyme activity by the removal of enzyme-bound zinc. At saturating levels of DNA, the enzyme was much less inhibited by 1,10-phenanthroline, suggesting that enzyme-bound zinc interacts with DNA. Scrutton et al. (1971) demonstrated that *E. coli* RNA polymerase was a zinc metalloenzyme containing 2 g-atom of zinc per mol. Since 1,10-phenanthroline did not remove zinc from the polymerase, the observed inhibition is probably due to the formation of an enzyme–zinc–phenanthroline ternary complex. Additional evidence presented by these authors suggests that 1,10-phenanthroline inhibits RNA chain initiation. When the chelator was added before the other reaction components, inhibition was instantaneous. On the other hand, when 1,10-phenanthroline was added after the other reaction components, inhibition occurred only after a lag period. Secondly, 1,10-phenanthroline prevented the binding of GTP to the initiation site in equilibrium dialysis experiments.

Eukaryotic RNA polymerases are also zinc metalloenzymes. Valenzuela et al. (1973) first reported inhibition of rat liver RNA polymerases I and II and sea urchin polymerases I, II, and III by 1,10-phenanthroline but did not directly demonstrate the presence of zinc in these enzymes. *E. gracilis* RNA polymerase II has recently been shown to contain approximately 2 g-atoms of zinc per mol (Falchuk et al., 1976). Inhibition by 1,10-phenanthroline was instantaneous and reversible, suggesting that the inhibition is due to the formation of an enzyme–zinc–phenanthroline ternary complex. Recently yeast nuclear RNA polymerases I, II, and III have been found to be zinc metalloenzymes (Auld et al., 1976; Lattke and Weser, 1976; Wandzilak and Benson, 1977). Zinc is tightly bound to these polymerases, and inhibition by metal chelating agents suggests that zinc is involved in the catalytic process. Polymerase I was found to contain 2.4 g-atoms of zinc based on a molecular weight of 650 000 (Auld et al., 1976) and polymerase II was found to contain 0.98 g-atom of zinc based on a molecular weight of 460 000 (Lattke and Weser, 1976). Additional data presented by these researchers suggest that inhibition by 1,10-phenanthroline is due to the formation of a ternary complex which blocks the catalytic reaction.

In this paper we report our method for purification of yeast RNA polymerase III by a different method from that recently reported by Hager et al. (1977) and extend our earlier observations on this zinc metalloenzyme (Wandzilak and Benson, 1977). We have now determined that polymerase III contains 2 g-atoms of zinc per mol. Zinc was not removed by treatment of the native enzyme with chelating agents but was removed when the enzyme was denatured. In addition enzyme-bound zinc did not exchange with free zinc.

Experimental Procedures

Materials. Phosphocellulose P11 and DE-81 ion exchange discs were obtained from Whatman, Inc. DEAE-Sephadex A-25, Sephadex G-25, Chelex-100, cyanogen bromide activated Sepharose 4B, nucleotides, calf thymus DNA, Trizma, polyvinylpyrrolidone (PVP-40), 1,10-phenanthroline, catalase, and yeast alcohol dehydrogenase were purchased from Sigma Chemical Co. β-Galactosidase was a Worthington preparation.

† From the Department of Biochemistry, Schools of Dentistry and Medicine, University of Louisville, Louisville, Kentucky 40232. *Received August 10, 1977.* Supported by GRS funds of the University of Louisville School of Medicine, and by a Homberger Memorial Fellowship from the Department of Biochemistry to T.M.W.

0006-2960/78/0417-0426$01.00/0

7,8-Benzoquinoline was purchased from Aldrich Chemical Co., UltraPure ammonium sulfate from Schwarz/Mann, Omnifluor from New England Nuclear, and zinc sulfate standard for atomic absorption spectroscopy from J. T. Baker. [5-^{3}H]UTP (20–25 Ci/mmol) and $^{65}ZnCl_2$ (0.26 mCi/μg) were obtained from Amersham/Searle.

Buffers. Buffer A is 10 mM Tris-Cl (pH 7.9, measured at 25 °C), 0.1 mM EDTA, 1.0 mM 2-mercaptoethanol, 25% (v/v) glycerol. Buffer B is the same solution lacking glycerol. The ammonium sulfate concentration in the buffers was estimated from the conductivity measured with a Radiometer CDM2 meter.

Resin Preparation. Phosphocellulose was washed with 0.5 N NaOH, then with distilled water, and then with 0.5 N HCl. After a final wash with distilled water, the resin was suspended in buffer A and the pH was adjusted to 7.9. DEAE-Sephadex and Chelex-100 were prepared in the same manner. DNA-Sepharose was prepared by allowing cyanogen bromide activated Sepharose 4B to react with heat denatured calf thymus DNA (type V) as described by Poonian et al. (1971). The resin contained approximately 2.4 A_{260} units of DNA per mL as determined by the loss of A_{260} after reaction with the activated Sepharose.

Yeast Cells. Wild type *Saccharomyces cerevisiae,* isolated from a commercial source, were grown in a New Brunswick Magnaferm fermentor at 32 °C with vigorous aeration to late log phase and were harvested using a Sharples centrifuge. The growth medium contained 1% glucose, 1% lactate (pH 4.5), 0.3% Difco Yeast Nitrogen Base. Cell yield was normally 10–20 g/L (wet weight).

RNA Polymerase Assays. The RNA polymerase activity was measured in a final volume of 50 μL. Each assay contained 60 mM Tris-Cl (pH 7.9), 1.0 mM 2-mercaptoethanol, 1.0 mM $MnCl_2$, 5 mM KCl, 0.05 mM EDTA, 12.5% (v/v) glycerol, 50 μg/mL heat denatured calf thymus DNA (type I), 0.05 mM UTP, 0.5 μCi of [^{3}H]UTP, and 0.1 mM each ATP, CTP, and GTP. After incubation at 30 °C for 20 min, the reaction was terminated by pipetting a 40-μL aliquot onto a disc of Whatman DE-81 ion-exchange paper. Unreacted [^{3}H]UTP was removed by washing the discs seven times with 5% disodium phosphate. The discs were then washed twice in distilled water, twice in 95% ethanol, once in diethyl ether, and air dried. Radioactive RNA on the disc was measured in a Nuclear Chicago Mark II scintillation counter in toluene containing 4 g/L Omnifluor. One unit of polymerase activity corresponds to the incorporation of 1 nmol of UMP into RNA under these conditions. For calculation of the specific activity, protein was measured by the procedure of Lowry et al. (1951) using bovine serum albumin as the standard.

Enzyme activity in a polyacrylamide gel was determined by slicing the gel into 2-mm segments. Each segment was diced and placed in the well of a tissue culture tray. Standard assay mixture (50 μL) was then added. After incubation at 30 °C for 60 min, the reaction was terminated by inserting a rolled disc of Whatman DE-81 into each well and allowing the liquid to be absorbed. The discs were then processed and counted as described above.

Zinc Analyses. An Instrumentation Laboratories atomic absorption spectrophotometer (Model 253) was used according to standard procedures for zinc determinations. Zinc standards prepared in the same buffer as the protein sample were aspirated into the flame and the absorbance at 213.8 nm was recorded. The zinc content of the protein sample was determined from the standard curve. All glassware used for the preparation of zinc standards was cleaned in HNO_3:H_2SO_4 (1:1) prior to use. The solutions were prepared using glass distilled water that had been made "metal free" by passing it through a column of Chelex-100. Solutions were stored in polyethylene bottles. ^{65}Zn was measured using a Nuclear Chicago γ counter (Model 1185).

TABLE I: Purification of Yeast RNA Polymerase III.[a]

Fraction	Protein (mg)	Spec act.[b] (units/mg)
Nuclear extract	4800	*c*
Phosphocellulose	620	*c*
DEAE-Sephadex	12.0	2.5
DNA-Sepharose	3.2	37.4[d]

[a] Four hundred grams of cells was used. [b] Measured at 0.1 M $(NH_4)_2SO_4$. [c] Not reported since polymerases I and II were also present. [d] When assayed at 0.6 mM ATP, CTP, and GTP, and 0.1 mM UTP, the specific activity was 500 units/mg.

Polyacrylamide Gel Electrophoresis. Disc electrophoresis in 5% polyacrylamide gels was conducted using the barbital buffer (system 6) described by Maurer (1971). The system was modified so that the gels contained 10% glycerol and 5 mM 2-mercaptoethanol. Gels were either stained with 0.04% Coomassie blue in methanol:acetic acid:water (5:1:5) and destained by diffusion in a solution of 50 mL of ethanol, 75 mL of acetic acid, and 875 mL of water, or were sliced into 2-mm segments for determination of RNA polymerase activity.

Glycerol Gradient Centrifugation. Sedimentation velocity centrifugation (Martin and Ames, 1961) in a 10–30% glycerol gradient was used to estimate the molecular weight of polymerase III. The protein standards used were yeast alcohol dehydrogenase (150 000), beef liver catalase (240 000), and *E. coli* β-galactosidase (520 000). The activity of these enzymes was determined as described by Vallee and Hoch (1955), Beers and Sizer (1952), and Craven et al. (1965), respectively.

Results

Purification of RNA Polymerase III. A summary of the purification of RNA polymerase III is presented in Table I. This method, described in detail below, gives a high yield of essentially pure RNA polymerase III. All steps were conducted at 4 °C.

Nuclear Extract. The harvested cells (400 g) were suspended in 400 mL of buffer containing 0.04 M Tris-Cl (pH 7.9), 0.5 M sorbitol, 2 mM EDTA, 20% (v/v) glycerol, and 10% (w/v) polyvinylpyrrolidone. The cells were lysed in a modified French Pressure Cell (Bhargava and Halvorson, 1971). The yeast suspension was frozen by placing the Cell in dry ice/ethanol and was extruded from the Cell at 10 000–15 000 psi. Yeast cell lysis was normally 60%. Intact nuclei and unbroken cells were collected by centrifugation (10 min at 1020*g*). The nuclei were lysed by suspending the pellet in 400 mL of buffer A containing 0.3 M ammonium sulfate. After treatment in a blender to reduce the viscosity, the extract was diluted with 800 mL of buffer A and centrifuged for 30 min at 27 000*g*. The supernatant was dialyzed against buffer A to reduce the ammonium sulfate concentration to less than 0.05 M and was used for enzyme purification.

Treatment with Phosphocellulose. RNA polymerases were adsorbed onto phosphocellulose essentially as described by Buhler et al. (1974). The dialyzed nuclear extract (1.6 L) was added to 400 g (wet weight) of phosphocellulose. The slurry was stirred for 30 min and collected by filtration in a Buchner funnel. The resin was then washed four times by suspending it in 800 mL of buffer A containing 0.05 M ammonium sulfate,

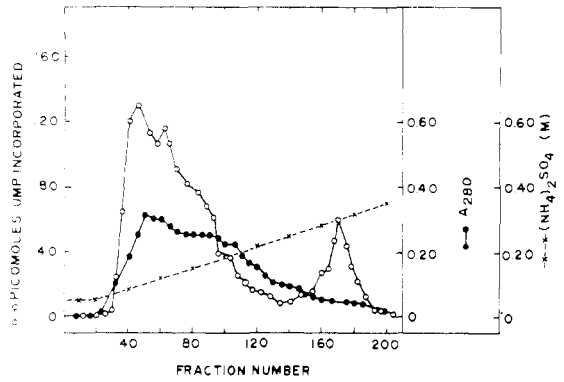

FIGURE 1: DEAE-Sephadex chromatography. The dialyzed sample after treatment with phosphocellulose was applied to a 250-mL DEAE-Sephadex column equilibrated with buffer A. The multiple polymerases were resolved with a 1.5-L gradient of 0.05 to 0.50 M ammonium sulfate in buffer A. Fractions of 6 mL were collected and assayed for A_{280} (●), RNA polymerase activity in a 25-μL aliquot (○), and ammonium sulfate concentration (X).

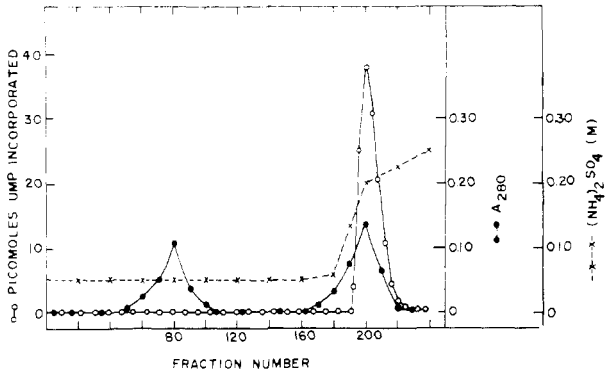

FIGURE 2: DNA-Sepharose chromatography. The dialyzed sample of polymerase III from the DEAE-Sephadex column was applied to a 150-mL column of DNA-Sepharose equilibrated with buffer A. After the column was washed with buffer A containing 0.05 M ammonium sulfate to remove the nonadsorbed protein, polymerase III was eluted with buffer A containing 0.25 M ammonium sulfate. Fractions of 3.5 mL were collected and assayed for A_{280} (●), RNA polymerase activity in a 25-μL aliquot (○), and ammonium sulfate concentration (X).

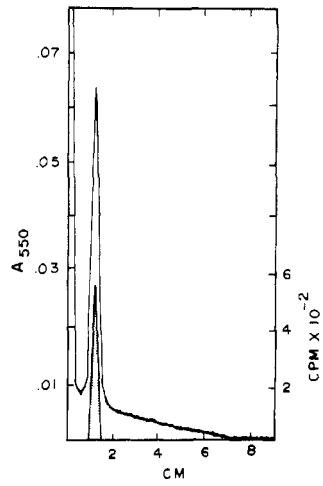

FIGURE 3: Polyacrylamide gel electrophoresis. The enzyme (8 μg of protein) purified through DNA-Sepharose chromatography was analyzed by electrophoresis in parallel 5% polyacrylamide gels as described in Experimental Procedures. One gel was stained with Coomassie blue to locate the protein by its absorbance at 550 nm (—) and the other gel was assayed for enzyme activity (shaded area). After correcting for the increase in size of the stained gel, the patterns were superimposed. Direction of electrophoresis is from left to right.

stirring for 30 min, and collecting it by filtration. RNA polymerases were eluted by suspending the resin in 600 mL of buffer A containing 0.6 M ammonium sulfate and stirring for 30 min. The filtrate contained the RNA polymerase activity.

DEAE-Sephadex Chromatography. The filtrate from above was dialyzed against buffer A to reduce the ammonium sulfate concentration to less than 0.05 M, and then loaded onto a column of DEAE-Sephadex A-25. The multiple polymerases were resolved with a linear salt gradient in buffer A (Figure 1). Polymerase III was eluted from the column at approximately 0.3 M ammonium sulfate.

DNA-Sepharose Chromatography. Fractions containing polymerase III activity were pooled and concentrated in an Amicon concentrator (PM-10 membrane) until the A_{280} was approximately 0.5. The sample was dialyzed against buffer A to reduce the ammonium sulfate concentration to less than 0.05 M and loaded onto a column of DNA-Sepharose. After washing the column to remove the nonadsorbed protein, polymerase III was eluted with buffer A containing 0.25 M ammonium sulfate (Figure 2). Some protein eluted from the column with the high salt wash that did not contain significant RNA polymerase activity. This may be an unrelated contaminant or inactive RNA polymerase which has a lower affinity for the DNA. This protein was discarded. The fractions containing polymerase activity were pooled and concentrated as described above. The specific activity of the enzyme was fairly low when assayed with our standard conditions. However, when the activity was measured in the presence of 0.6 mM ATP, CTP, and GTP, and 0.1 mM UTP, the specific activity was 500 units/mg. This specific activity is identical with that reported by Hager et al. (1977) for their purified yeast RNA polymerase III.

Polyacrylamide Gel Electrophoresis. The final product was analyzed by electrophoresis in 5% polyacrylamide gels. The results (Figure 3) showed a single Coomassie blue staining band in the gel and some Coomassie blue staining material that did not enter the gel. When the companion gel was assayed for polymerase activity, the activity was coincident with the protein band in the gel. The material at the top of the gel may be inactive, aggregated polymerase III or a high molecular weight contaminant.

Molecular Weight. Sedimentation velocity centrifugation in a 10–30% glycerol gradient was used to estimate the molecular weight of the enzyme. The gradient contained 0.35 M NH_4Cl to prevent enzyme aggregation. The result (Figure 4) indicated that the molecular weight of polymerase III was approximately 380 000.

Inhibition by 1,10-Phenanthroline. By analogy with other nucleotidyl transferases, it was reasonable to believe that yeast RNA polymerase III was also a zinc metalloenzyme. Inhibition of enzyme activity by a metal chelating agent provides indirect evidence that a metal ion is essential to the catalytic process. Accordingly, the effect of 1,10-phenanthroline on the purified yeast RNA polymerase III was determined. The data of Figure 5 show that polymerase III was inhibited more than 50% at 0.1 mM and essentially 100% at 1.0 mM 1,10-phenanthroline.

This enzyme requires a divalent metal ion (either Mn^{2+} or Mg^{2+}) for activity. The optimum Mn^{2+} concentration for polymerase activity is 1 mM. Since 1,10-phenanthroline will also chelate Mn^{2+}, the observed inhibition could have been due to binding of this essential cation. In order to eliminate this possibility, enzyme assays were supplemented with additional $MnCl_2$ equimolar to the 1,10-phenanthroline concentration.

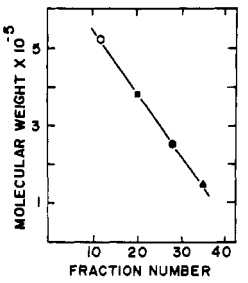

FIGURE 4: Molecular weight of polymerase III. The gradients for the sedimentation velocity centrifugation were 10 to 30% glycerol in buffer B containing 0.35 M ammonium chloride. Polymerase III and the protein standards prepared in buffer B containing 5% glycerol were combined and layered onto a gradient tube. The sample was centrifuged at 4 °C in a Beckman L3-50 centrifuge at 40 000 rpm for 14 h in the SW-41 Ti rotor. Fractions of 0.35 mL were collected and assayed for β-galactosidase (○), catalase (●), yeast alcohol dehydrogenase (▲), and RNA polymerase (■) as described in Experimental Procedures.

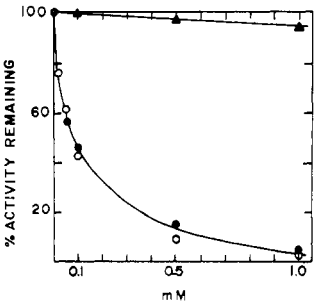

FIGURE 5: Inhibition of RNA polymerase III by 1,10-phenanthroline. Purified RNA polymerase III was preincubated 20 min at the indicated concentration of 1,10-phenanthroline. Identical results were obtained when the preincubation was 10 min. Assay mix containing the same 1,10-phenanthroline concentration was used for measurement of polymerase activity. Activities at 1 mM $MnCl_2$ in the presence of the indicated concentration of 1,10-phenanthroline (○), or at 1 mM $MnCl_2$ plus additional $MnCl_2$ equimolar to 1,10-phenanthroline (●) are expressed relative to the activity in the absence of inhibitor (defined as 100%). Also shown are the activities at 1 mM $MnCl_2$ in the presence of the indicated concentration of 7,8-benzoquinoline (▲).

No change in the inhibition profile was observed under these conditions (Figure 5). These results indicate that 1,10-phenanthroline inhibits enzyme activity by chelation of an intrinsic metal ion of the polymerase. This conclusion is strengthened by the demonstration that 7,8-benzoquinoline, a nonchelating structural analogue of 1,10-phenanthroline, did not inhibit the polymerase activity (Figure 5).

Zinc Stoichiometry. Atomic absorption spectroscopy was used to directly demonstrate the presence of zinc in the polymerase and to establish the zinc-protein stoichiometry. A standard curve was determined using zinc standards prepared in the same buffer as the protein. The average of two absorbance measurements on each protein solution was used to determine the zinc concentration from the standard curve. The stoichiometry was calculated using 380 000 as the molecular weight of the enzyme. Typical results are shown in Table II. Based on these data, the enzyme contains 2 g-atoms of zinc per mol.

Specific Association of Zinc and Enzyme. The above data suggest that zinc is specifically and tightly associated with RNA polymerase III since it is retained through chromatographic treatments that would be expected to remove loosely associated metal ions. Direct evidence for the specific association of zinc with the polymerase was obtained using gel filtration and a chelating resin. A sample of purified enzyme was passed through a 20-mL column of Sephadex G-25. The results indicated that protein, enzymatic activity, and zinc comigrated on the column and that the zinc-enzyme stoichiometry was not altered (data not shown). The data of Figure 6 demonstrate that this column was capable of resolving RNA polymerase activity from non-protein-bound zinc.

Additional evidence for a specific association of zinc with the polymerase was obtained by treating the enzyme with Chelex-100. The protein was incubated at 4 °C for 2 h with a threefold molar excess of zinc sulfate and then passed through a 0.5-mL column of Chelex-100. The zinc binding capacity of the column was at least 100 times greater than the amount of zinc present in the sample. Neither the zinc content nor the RNA polymerase activity was altered by this treatment (Table II).

These data indicate that the zinc is tightly associated with the polymerase and may only be removed by denaturing the protein. To confirm that zinc could be removed from denatured enzyme, a sample of enzyme was denatured by heating to 100 °C for 3 min in the presence of 1% sodium dodecyl sulfate. These conditions would be expected to completely denature the enzyme and allow removal of noncovalently bound zinc.

TABLE II: RNA Polymerase III Activity and Zinc Content.

	Rel act. (%)	Zinc content (g-atoms/mol)
Before Chelex-100 chromatography	100	2.09
After Chelex-100 chromatography	100	2.32
Before dialysis vs. 1,10-phenanthroline	100	2.01
After dialysis vs. 1,10-phenanthroline	13	1.82
After dialyzing out 1,10-phenanthroline	68	Not determined

The denatured enzyme was then dialyzed against 30 volumes of buffer A containing 0.01 mM 1,10-phenanthroline. The buffer solution was changed three times at 4-h intervals. After dialysis the zinc content of the protein was 0.14 g-atom per mol. Thus, greater than 90% of the enzyme-bound zinc can be removed from denatured enzyme.

Mechanism of 1,10-Phenanthroline Inhibition. 1,10-Phenanthroline may inhibit enzyme activity by the removal of an essential zinc atom from the polymerase, or by the formation of an enzyme-zinc-phenanthroline ternary complex which blocks catalytic activity. In order to determine the mode of inhibition, the enzyme was dialyzed at 4 °C against 20 volumes of buffer A containing 1.0 mM 1,10-phenanthroline. The buffer solution was changed three times at 4-h intervals. The zinc content and enzyme activity were subsequently measured. No significant change in the zinc content of the polymerase was observed, but enzyme activity was reduced to 13% of the original value (Table II). Enzyme activity was restored to 68% of the original value when the inhibitor was removed by dialysis against 300 volumes of buffer A which was changed twice at 6-h intervals. These experiments established that the chelator did not remove protein-bound zinc and that the inhibition was reversible. These results strongly suggest that an enzyme-zinc-phenanthroline ternary complex is responsible for the inhibition.

Zinc Exchange Experiments. The data indicate that enzyme-bound zinc shows little, if any, tendency to dissociate from the protein. It was possible, however, that the protein-bound zinc could exchange directly with zinc in solution. Radioactive zinc was used as a sensitive probe for any exchange reaction by incubating purified enzyme with varying amounts of radioactive zinc. Protein and free zinc were then resolved

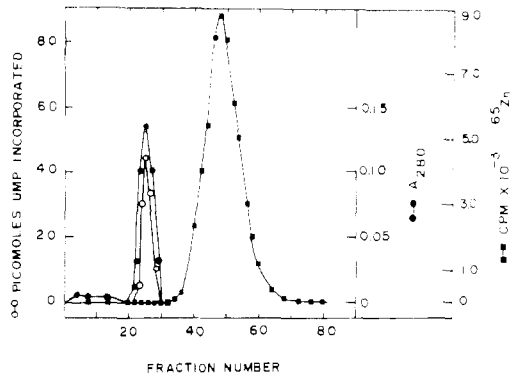

FIGURE 6: Zinc exchange. Polymerase III (80 μg, containing 0.025 μg zinc) was incubated for 24 h at 25 °C with 0.022 μg radioactive zinc. The sample was then chromatographed on a 20-mL Sephadex G-25 column equilibrated with zinc-free buffer A. Fractions of 0.25 mL were collected and assayed for A_{280} (●), enzyme activity in a 25 μL aliquot (○), and ^{65}Zn (■).

by gel filtration using Sephadex G-25. As seen in Figure 6, when protein was incubated with an equivalent amount of zinc at 25 °C for 24 h, no detectable zinc exchange occurred. Similar results (data not shown) were obtained when the incubation was at 4 °C. In addition no detectable zinc exchange occurred when the protein was incubated at 4 °C for 24 h with an amount of free zinc 10 times greater than or 100 times lower than that present in the protein (data not shown).

Discussion

We have developed a rapid and reliable technique for the purification of yeast RNA polymerase III from isolated nuclei. Adsorption onto phosphocellulose has been used in other laboratories (Valenzuela et al., 1975; Buhler et al., 1974) and is a very useful first step in the purification of large quantities of RNA polymerases. The nuclear extract from several hundred grams of yeast can be processed in a short time, thus avoiding time-consuming chromatographic steps early in the purification. Phosphocellulose treatment also removes nucleic acids which seriously interfere with chromatography on DEAE-Sephadex and is superior to the use of protamine sulfate which often causes loss of the polymerases (Hager et al., 1977). DEAE-Sephadex effectively resolves the multiple polymerase activities in a single chromatographic step. RNA polymerase III elutes from the column at about 0.3 M ammonium sulfate and is well separated from polymerases I and II and from the bulk of the protein applied to the column. Final purification is achieved by affinity chromatography on DNA-Sepharose.

This purification scheme gives a high yield of essentially pure enzyme. Analyses on polyacrylamide gels revealed a single protein band which contained enzyme activity. Protein that did not enter the gel matrix may be aggregated enzyme or a high molecular weight contaminant. The specific activity was identical with that recently reported by Hager et al. (1977), who purified yeast RNA polymerase III by a different procedure. The specific activity and the gel electrophoresis data suggest that the polymerase obtained in the present study was approximately 90% pure.

The data obtained with yeast RNA polymerase III permit its classification as a zinc metalloenzyme. Inhibition by the chelator 1,10-phenanthroline, but not by the nonchelating structural analogue 7,8-benzoquinoline, provides indirect evidence for the metalloenzyme nature of the polymerase. Inhibition of 50% of the activity at 0.1 mM is in the range reported by other groups for RNA polymerases from *E. coli* (Scrutton et al., 1971), *E. gracilis* (Falchuk et al., 1976), and yeast (Auld et al., 1976; Lattke and Weser, 1976). The possibility that the inhibition was due to chelation of Mn^{2+} was ruled out. Under conditions where the free Mn^{2+} was always greater than 1 mM, the inhibition profile was not altered. These data indicate that the inhibition is due to the chelation of an intrinsic metal ion of the polymerase. This inhibition appears to be due to the formation of a reversible enzyme–zinc–phenanthroline ternary complex since zinc was not removed from the protein by the inhibitor and the enzyme activity was restored when the inhibitor was removed by dialysis. Other RNA polymerases seem to be inhibited by a similar mechanism (Scrutton et al., 1971; Falchuk et al., 1976; Auld et al., 1976; Lattke and Weser, 1976).

Direct evidence that zinc is the intrinsic metal ion was provided by atomic absorption spectroscopy. Supportive evidence was provided by comigration of zinc, protein, and enzyme activity after chromatography on Sephadex G-25 as well as Chelex-100. From the quantity of zinc measured by atomic absorption and the molecular weight of 380 000 measured by sedimentation velocity centrifugation, yeast RNA polymerase III contains 2 g-atoms of zinc per mol. This value compares favorably with the value of 2.2 reported by Falchuk et al. (1976) for *E. gracilis* polymerase II, and the value of 2.4 reported by Auld et al. (1976) for yeast RNA polymerase I, but is twice that reported by Lattke and Weser (1976) for yeast polymerase II.

The zinc bound to polymerase III is very tightly associated with the protein. It was not removed by gel filtration, by Chelex-100 resin, or by dialysis against buffer containing 1,10-phenanthroline. However, the zinc can be removed by dialysis when the protein is denatured. The protein-bound zinc also did not exchange with free zinc under a variety of experimental conditions. These data suggest that the zinc shows little, if any, tendency to dissociate from the native enzyme. The other eukaryotic RNA polymerases (Falchuk et al., 1976; Auld et al., 1976; and Lattke and Weser, 1976) and the *E. coli* enzyme (Scrutton et al., 1971) also appear to tightly bind intrinsic zinc atoms. In contrast zinc is easily removed by chelating agents from *E. coli* DNA polymerase (Slater et al., 1971) and bacteriophage T_7 RNA polymerase (Coleman, 1974).

The functional role of the zinc in yeast RNA polymerase III is not known. Zinc may be involved in the maintenance of subunit structure or in the catalytic reaction or in both. The inhibition of enzyme activity by a metal chelator suggests that zinc has at least some catalytic role. Zinc may be responsible for the binding of nucleoside triphosphates. Scrutton et al. (1971) found that the binding of GTP to the initiation site of *E. coli* RNA polymerase was inhibited by 1,10-phenanthroline. Zinc may also be involved in binding of the DNA template. In *E. coli* RNA polymerase, zinc has been found to be associated with the β′ subunit (Wu et al., 1977), which is believed to be responsible for the binding of the DNA template (Fukuda and Ishihama, 1974). Of interest in this regard is the earlier observation of Slater et al. (1971) that 1,10-phenanthroline was a much less effective inhibitor of *E. coli* DNA polymerase at saturating levels of DNA.

Zinc could also have a more direct role in the catalytic reaction. Zinc may serve as a Lewis acid to increase the nucleophilic character of the 3′-hydroxyl group of the terminal ribose (Auld et al., 1976). This would permit a more facile attack by the hydroxyl group at the α-phosphoryl group of the nucleotide to be attached to the nascent RNA chain.

All three of the yeast nuclear RNA polymerases have been demonstrated to be zinc metalloenzymes. Of importance now

is the elucidation of the specific protein ligands to the zinc atoms and how the zinc atoms participate in the catalytic reaction and in the protein structure.

Acknowledgments

We thank the Department of Pharmacology and Toxicology for the use of the Instrumentation Laboratories atomic absorption spectrophotometer and the Department of Physiology and Biophysics for the use of the Nuclear Chicago γ counter.

References

Auld, D. S., Kawaguchi, H., Livingston, D. M., and Vallee, B. L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2091.
Auld, D. S., Kawaguchi, H., Livingston, D. M., and Vallee, B. L. (1975), *Biochem. Biophys. Res. Commun. 62,* 296.
Auld, D. S., Atsuya, I., Campino, C., and Valenzuela, P. (1976), *Biochem. Biophys. Res. Commun. 69,* 548.
Beers, R. F., Jr., and Sizer, I. W. (1952), *J. Biol. Chem. 195,* 133.
Bhargava, M. M., and Halvorson, H. O. (1971), *J. Cell Biol. 49,* 423.
Buhler, J.-M., Sentenac, A., and Fromageot, P. (1974), *J. Biol. Chem. 249,* 5963.
Coleman, J. E. (1974), *Biochem. Biophys. Res. Commun. 60,* 641.
Craven, G. R., Steers, E., Jr., and Anfinsen, C. B. (1965), *J. Biol. Chem. 240,* 2468.
Falchuk, K. H., Mazus, B., Ulpino, L., and Vallee, B. L. (1976), *Biochemistry 15,* 4468.
Fukuda, R., and Ishihama, A. (1974), *J. Mol. Biol. 87,* 523.
Hager, G. L., Holland, M. J., and Rutter, W. J. (1977), *Biochemistry 16,* 1.
Lattke, H., and Weser, U. (1976), *FEBS Lett. 65,* 288.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236,* 1372.
Maurer, H. J. (1971), Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis, New York, N.Y., DeGruyter.
Poiesz, B. J., Seal, G., and Loeb, L. A. (1974), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2497.
Poonian, M. S., Schlaback, A. J., and Weissbach, A. (1971), *Biochemistry 10,* 424.
Scrutton, M. C., Wu, W. C., and Goldthwait, D. A. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2497.
Slater, J. P., Mildvan, A. S., and Loeb, L. A. (1971), *Biochem. Biophys. Res. Commun. 44,* 37.
Todd, W. R., Elvejhem, C. A., and Hart, E. B. (1934), *Am. J. Physiol. 107,* 146.
Valenzuela, P., Morris, R. W., Faras, A., Levinson, W., and Rutter, W. J. (1973), *Biochem. Biophys. Res. Commun. 53,* 1036.
Valenzuela, P., Weinberg, F., Bell, G., and Rutter, W. J. (1975), *J. Biol. Chem. 251,* 1464.
Vallee, B. L. (1959), *Physiol. Rev. 39,* 443.
Vallee, B. L. (1976), *Trends Biochem. Sci. 1,* 88.
Vallee, B. L., and Hoch, F. L. (1955), *Proc. Natl. Acad. Sci. U.S.A. 41,* 327.
Wandzilak, T. M., and Benson, R. W. (1977), *Biochem. Biophys. Res. Commun. 76,* 247.
Wu, C. W., Speckhard, D., and Wu, F. Y.-H. (1977), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36,* 882.

Possible Site-Specific Reagent for the General Amino Acid Transport System of *Saccharomyces cerevisiae*†

Fred S. Larimore and Robert J. Roon*

ABSTRACT: The general amino acid transport system of *Saccharomyces cerevisiae* functions in the uptake of neutral, basic, and acidic amino acids. The amino acid analogue *N*-δ-chloroacetyl-L-ornithine (NCAO) has been tested as a potential site specific reagent for this system. L-Tryptophan, which is transported exclusively by the general transport system, was used as a substrate. In the presence of glucose as an energy source, NCAO inhibited tryptophan transport competitively ($K_i = 80\ \mu M$) during short time intervals (1–2 min), but adding 100 μM NCAO to a yeast cell suspension resulted in a time-dependent activation of tryptophan transport during the first 15 min of treatment. Following the activation a time-dependent decay of tryptophan transport activity occurred. Approximately 80% inactivation of the system was observed after 90 min. When a yeast cell suspension was treated with NCAO in the absence of an energy source, an 80% inactivation of tryptophan transport occurred in 90 min. The inactivation was noncompetitive ($K_i \simeq 60\ \mu M$) and could not be reversed by the removal of the NCAO. Addition of a fivefold excess of L-lysine during NCAO treatment or prevented inactivation of tryptophan transport. Under parallel conditions of incubation, other closely related transport systems were not inhibited by NCAO.

The transport of amino acids in *Saccharomyces cerevisiae* is accomplished by a general amino acid transport system having a broad substrate specificity and by a number of specific transport systems having more stringent substrate specificities (Crabeel and Grenson, 1970; Gits and Grenson, 1967; Grenson et al., 1966, 1970; Jorris and Grenson, 1969). The maximal level of activity observed with the general amino acid transport system is 5- to 50-fold higher than that found with the various specific systems. Grenson and her associates, who initially

† From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received August 1, 1977.* This work was supported in part by Research Grant No. GM 23135 from the National Institutes of General Medical Sciences.

0006-2960/78/0417-0431$01.00/0