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Purification and Properties of Ribonucleic Acid Polymerase from Rat Liver Mitochondria†

Himansu Mukerjee and Anna Goldfeder*,‡

ABSTRACT: A DNA-dependent RNA polymerase was solubilized from mitochondrial membranes of rat liver and purified by differential and sucrose density gradient centrifugation and column chromatography on DEAE-Sephadex. The possibility of nuclear enzyme contamination of the mitochondrial enzyme preparation has been ruled out. The enzyme shows the requirement of Mn^{2+} and the presence of all four ribonucleoside triphosphates. It is completely insensitive to α-amanitin and is inhibited by rifampicin by 80% at 10 μg/ml. The enzyme activity is 80–90% dependent on added DNA template and both native calf-thymus DNA and mitochondrial DNA can serve as templates. The enzyme sediments at 4.3 S and appears to consist of one polypeptide chain with mol wt measured to be 66,000 on gel electrophoresis in the presence of sodium dodecyl sulfate.

Recent observations suggest that mitochondria possess a considerable degree of autonomy in their biogenesis (Slater *et al.*, 1968; Roodyn and Wilkie, 1968). It has been demonstrated in several organisms that mitochondria can also synthesize their own RNA (Wintersberger, 1964; Luck and Reich, 1964; Neubert and Helge, 1965; Kalf, 1964; Suyama and Eyer, 1968). Tsai *et al.* (1971) have solubilized and purified mitochondrial RNA polymerase from yeast and reported that it differs from the corresponding nuclear enzyme in lacking inhibition by α-amanitin.

RNA polymerase activity in rat liver mitochondria, either in the intact organelle or in the solubilized form, has also been reported by several investigators (Saccone and Gadaleta, 1970; Wintersberger and Wintersberger, 1970; Gadaleta *et al.*, 1970; Shmerling, 1969). Shmerling (1969) and Gadaleta *et al.* (1970) found that RNA polymerase activity in intact rat liver mitochondria was highly sensitive to rifampicin, whereas nuclear RNA polymerase activity was not.

Recently, Reid and Parsons (1971) reported the solubilization and partial purification of RNA polymerase from rat liver mitochondria. But these investigators found that the partially purified enzyme was only slightly inhibitable by rifampicin. This paper describes the isolation of membrane bound mitochondrial RNA polymerase from rat liver, its solubilization, and purification. This purified hepatic mitochondrial RNA polymerase was found to be highly sensitive to inhibition by rifampicin.

Materials

Rifampicin, a rifamycin derivative used for inhibition studies, was obtained from Calbiochem. α-Amanitin was a gift from Professor T. Wieland, Max-Planck Institute for Medical Research, Heidelberg, West Germany. All other biochemicals were obtained commercially.

† From the Cancer and Radiobiological Research Laboratory, Department of Health and Hospitals, Francis Delafield Hospital, New York, New York 10032, and the Department of Biology, New York University, New York, New York 10032. *Received February 21, 1973.* A preliminary account of parts of this work has appeared (Mukerjee and Goldfeder, 1972). This work was supported in part by grants from the National Institutes of Health and the Mildred Werner League for Cancer Research.

‡ Cancer and Radiobiological Research Laboratory.

TABLE I: Localization of Mitochondrial RNA Polymerase.[a]

Enzyme Fraction	Total Protein (mg)	Sp Act. (pmol of UMP/mg of Protein per 10 min)		Total Enzyme Units[b]	
		+Calf Thymus DNA	−Calf Thymus DNA	+Calf Thymus DNA	−Calf Thymus DNA
Sonicated suspension	19.6	10.2 ± 0.58	9.6 ± 0.6	200	188
Supernatant	11.4	3.65 ± 0.35	2.9 ± 0.22	42	33
Pellet (membrane)	7.4	17.1 ± 1.1	16.8 ± 0.88	126	125

[a] Isolated mitochondria were sonicated 60 sec at the maximum power setting of the Branson sonicator. The suspension was centrifuged 1 hr at 59,000*g* in a Spinco 40 rotor at 5°. The supernatant was removed and the pellet was resuspended in sucrose–Tris–EDTA buffer (see Methods). Each enzyme fraction (0.025 ml) was added to an assay mixture containing 1 μmol of $MgCl_2$, 25 μmol of Tris-HCl (pH 7.8), 1 μmol of mercaptoethanol, 1 μmol of ammonium sulfate, 0.04 μmol each of ATP, GTP, and CTP, 1 nmol of [^{3}H]UTP (250 Ci/mol or 1.03×10^5 cpm/nmol), ±20 μg of calf thymus DNA, and distilled water to 0.25 ml. The assays were incubated 10 min at 37°. The rest of the procedure is the same as described in the text. [b] A unit of enzyme is defined as the pmol of [^{3}H]UTP incorporated into RNA per mg of mitochondrial protein per 10 min.

Methods

Solubilization and Purification of RNA Polymerase. Mitochondria (220 mg of protein) were prepared and purified from rat liver by the method of O'Brien and Kalf (1967). About 20 mg/ml of mitochondrial suspension in 0.01 M Tris-HCl (pH 7.5) containing 0.01 M $MgCl_2$ was sonicated for 60 sec at the maximum power setting in a Branson sonicator. The sonicated suspension was centrifuged at 59,000*g* for 1 hr. Since 70–75% of RNA polymerase (enzyme units, Table I) was found in the mitochondrial membrane pellets, the enzyme was solubilized from the membrane fraction. The membrane pellet (72 mg of protein) in 3 ml of 0.25 M sucrose–0.01 M Tris-HCl (pH 7.8)–2 mM EDTA–5% glycerol–0.1 mM dithiothreitol was homogenized 50 times in motor-driven Teflon glass homogenizer. The suspension was incubated with DNase I (20 μg/ml) for 30 min at 4°. Potassium deoxycholate (1 mg of deoxycholate/mg of protein) was added to the suspension and the mixture was stirred for another 30 min at 4°. The suspension was centrifuged at 20,000*g* for 10 min. The pellet was discarded and the supernatant was recentrifuged first at 105,000*g* for 30 min and finally at 150,000*g* for 60 min. The final supernatant contained a colorless upper portion and a yellow colored lower portion which were separated as fractions I and II, respectively. The specific activities of RNA polymerase of both fractions were almost the same.

However, because of its higher protein concentration, fraction II was selected for further purification. One-milliliter aliquots of fraction II (12–15 mg of protein) were layered over 28 ml of 10–40% linear sucrose gradients containing 0.01 M Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol and centrifuged at 52,000*g* for 16 hr in a Spinco SW-25 rotor at 4°. The tubes were punctured at the bottom and 1-ml fractions were collected from the gradients and assayed for RNA polymerase activity. The contents of the tubes of the first elution peak were pooled and an aliquot was dialyzed against 0.05 M Tris-HCl (pH 7.8) buffer containing 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 5% glycerol. The dialyzed enzyme preparation was further incubated successively with DNase (20 μg/ml) for 16 hr at 4° and for another 2 hr at 30° with RNase (7.5 μg/ml). Then 4-ml linear gradients of 10–30% glycerol containing 0.05 M Tris-HCl (pH 8), 0.1 mM dithiothreitol, and 0.5 M KCl were layered with 0.5 ml of the dialyzed, nuclease-treated enzyme fraction and centrifuged at 105,000*g* for 3 hr in a Spinco SW-39 rotor at 4°. The tubes were punctured at the bottom and gradients were fractionated into 0.5-ml fractions and assayed for RNA polymerase activity.

An aliquot of the original sucrose density gradient purified enzyme (prior to RNase and DNase treatment) was dialyzed against 0.05 M ammonium sulfate solution containing 25% glycerol, 0.05 M Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol (TGMED buffer).[1] This dialyzed enzyme was incubated with DNase and RNase as described previously, and applied to a DEAE-Sephadex A-50 column (1 × 12 cm) which was equilibrated with TGMED–0.05 M $(NH_4)_2SO_4$ buffer and eluted according to the method of Roeder and Rutter (1969). One-milliliter fractions were collected and assayed for RNA polymerase activity.

Assay for RNA Polymerase Activity. RNA polymerase was routinely measured as follows. Reaction mixtures (0.25 ml) contained 20 μg of calf thymus DNA (Sigma), 1 μmol of $MgCl_2$, 0.25 μmol of $MnCl_2$, 25 μmol of Tris-HCl (pH 7.9), 1 μmol of mercaptoethanol, 1 μmol of $(NH_4)_2SO_4$, 0.04 μmol each of ATP, GTP, and CTP, 1 nmol of [^{3}H]UTP (1.03×10^5 cpm/nmol), and 0.015–0.5 A_{280} unit of enzyme preparation. After incubation at 37° in a water bath for 10 min, 1 ml of a mixture of equal volumes of 10% Cl_3CCOOH, saturated sodium pyrophosphate, and sodium orthophosphate solutions was added to stop the reaction and the tube was cooled in ice for 10 min. Acid-precipitable radioactivity was collected on Millipore filters and washed four times with 10 ml of 5% Cl_3CCOOH containing 0.01 M sodium pyrophosphate. After drying, the filters were immersed in 10 ml of toluene scintillation solution and counted in a Nuclear-Chicago scintillation counter.

Sucrose Gradient Sedimentation. An aliquot of DEAE-Sephadex purified enzyme fraction (peak III in Figure 3) was dialyzed overnight against a buffer containing 25 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.55. Two hundred microliters (~100 μg of protein)

[1] Abbreviations used are: mtDNA, mitochondrial DNA; TGMED buffer, Tris, glycerol, magnesium chloride, EDTA, and dithiothreitol buffer; nRNA, nuclear RNA.

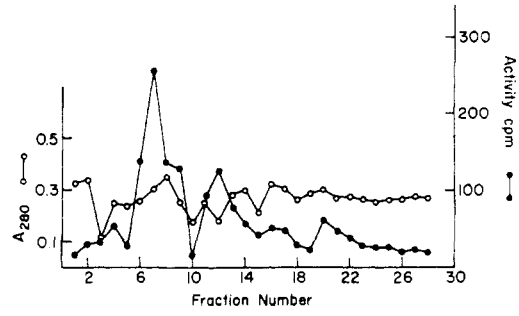

FIGURE 1: Sucrose density gradient centrifugation of potassium deoxycholate solubilized fraction of mitochondrial RNA polymerase. Details are described in the text. Aliquots (100 μl) of each fraction ((bottom first) were assayed for RNA polymerase activity.

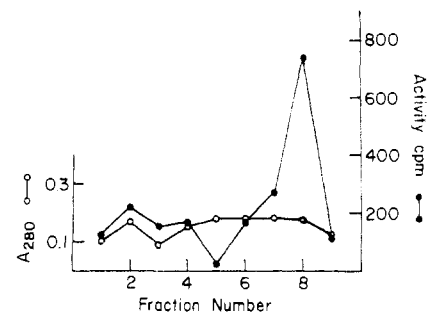

FIGURE 2: Rat liver mitochondrial RNA polymerase activity in a 10–31% glycerol gradient containng 0.5 M KCl, centrifuged at 105,000g for 3 hr in an SW-39 rotor. Aliquots (100 μl) of each fraction (bottom first) were assayed for RNA polymerase activity.

of the dialyzed enzyme preparation were applied to a linear sucrose gradient (5–20% w/v) prepared in dialyzing buffer and centrifuged in the SW-41 Spinco rotor at 41,000 rpm for 17 hr at 0°. At the end of the run, 0.5-ml fractions were collected starting from the top and assayed for RNA polymerase activity. For determination of the sedimentation coefficients of mtRNA[1] polymerase, the following proteins were used as standards: horse heart cytochrome *c* (2.1 S; Atlas and Farber, 1956), bovine serum albumin (4.3 S; Creeth, 1952), beef liver catalase (11.15 S; Samejima *et al.*, 1962).

Polyacrylamide Gel Electrophoresis. The subunit structure of DEAE-Sephadex purified enzyme (peak III in Figure 3) was studied by electrophoresis in 10% polyacrylamide gels in the presence of sodium dodecyl sulfate. Sodium dodecyl sulfate gel electrophoresis was carried out exactly as described by Weber and Osborn (1969). Horse heart cytochrome *c* (mol wt 13,400), ovalbumin (mol wt 44,000), and bovine serum albumin (mol wt 66,000) were used as standards.

Protein Determination. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Mitochondrial DNA. Mitochondrial DNA from rat liver was prepared by the method of Kalf and Grèce (1966).

Results

Purity of Mitochondria. Samples of fivefold washed mitochondrial pellets were examined in an electron microscope for purity. The numerous mitochondria seen in the field appeared intact. They differed in size and were filled with internal membranes (cristae). There was relatively little extraneous membranous material within the mitochondrial population.

Localization of Mitochondrial RNA Polymerase. As shown in Table I, 75% of the mitochondrial RNA polymerase is present in the membrane pellet. The lower specific activity in the sonicated suspension might be due to the presence of other extraneous proteins. The enzyme activity is not changed in the absence of exogenous DNA. This suggests that the crude enzyme is essentially saturated with endogenous DNA.

Purified Enzyme. The results of a typical purification of mitochondrial RNA polymerase from membrane fragments are shown in Table II. It was observed that much of the RNA polymerase activity was lost when the crude enzyme was precipitated by 30–50% $(NH_4)_2SO_4$ saturation of the deoxycholate solubilized fraction. The crude enzyme was therefore partially purified by sucrose density gradient centrifugation. The sucrose density gradient profile is shown in Figure 1. Two enzyme activity peaks were obtained. The fraction containing the higher activity in the first peak was subjected to further purification. In glycerol gradient purification, the enzyme activity appeared as a single peak (Figure 2).

When sucrose gradient purified enzyme (peak I of Figure 1) was applied to DEAE-Sephadex, four distinct activity peaks were obtained (Figure 3). Table III shows the sensitivity of these different peaks to antibiotics. Peak I is highly sensitive to α-amanitin and is therefore probably a nuclear contaminant. Peaks II and IV are insensitive to all the drugs. Peak III is highly sensitive to rifampicin and completely

TABLE II: Purification of Mitochondrial RNA Polymerase from Rat Liver.

Steps	Total Protein (mg)	Sp Act. (pmol of UMP/mg of Protein per 10 min)	Total Enzyme Units	Purification -Fold
1. Whole mitochondria	220	3.2	7×10^2	1
2. Homogenized membranes	72	15	10.8×10^2	4.7
3. Potassium deoxycholate[a] solubilized fraction II	36	5	1.8×10^2	1.6
4. Sucrose gradient peak (I)	5.4	31.5	1.7×10^2	9.8
5a. Glycerol gradient peak[b]	2.7	129.5	3.5×10^2	40.5
5b. DEAE-Sephadex peak (III)[b]	1.1	161	1.8×10^2	50.3

[a] Fraction II was obtained by differential centrifugation of potassium deoxycholate solubilized membranes. [b] In 5a an aliquot of the dialyzed, nuclease-treated enzyme fraction obtained from sucrose gradient peak I was layered with glycerol gradient, and in 5b a similar aliquot was applied to DEAE-Sephadex.

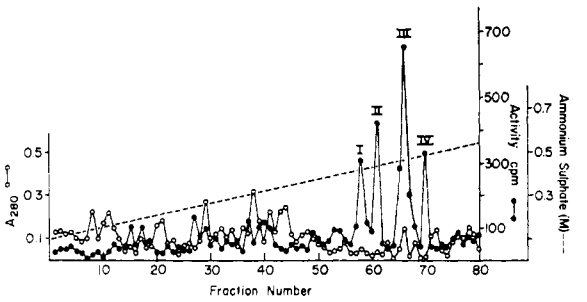

FIGURE 3: Mitochondrial RNA polymerase activity eluted from a DEAE-Sephadex column. The enzyme activities were eluted with a linear gradient of 0.1–0.5 M $(NH_4)_2SO_4$ in TMGED buffer. Aliquots (100 μl) of each fraction were assayed for RNA polymerase activity. Further details are described in the text.

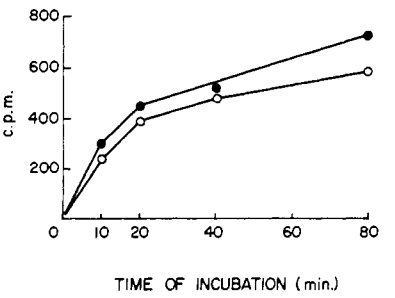

FIGURE 4: Kinetics of incorporation of UTP into RNA by rat liver mitochondrial RNA polymerase with different DNA templates. The standard reaction mixture is the same as described in the text except that DEAE-Sephadex, peak III enzyme fraction, A_{280} = 0.015, and 20 μg of mtDNA were used in each assay: (●) mtDNA; (○) calf thymus DNA.

sensitive to α-amanitin and most probably represents mitochondrial RNA polymerase.

Some of the properties of mitochondrial RNA polymerase purified either by glycerol gradient centrifugation or by DEAE-Sephadex chromatography are given in Table IV.

The DEAE-Sephadex peak III enzyme is completely insensitive to α-amanitin, whereas the less pure glycerol gradient sedimented enzyme is slightly sensitive to α-amanitin. This might be due to the contamination therein of the α-amanitin sensitive polymerase which is peak I of Figure 3. However, high rifampicin sensitivity has been observed in both cases. The enzyme requires a complement of all four ribonucleoside triphosphates and DNA template.

Both Mg^{2+} and Mn^{2+} are necessary for maximal activity of the enzyme. A threefold reduction of Mg^{2+} concentration results in about 30% loss of activity.

The DEAE-Sephadex purified enzyme appears to be more sensitive to inhibition by actinomycin D (80%). The inhibition of enzyme activity in the presence of actinomycin D further indicates that purified enzyme (peak III, Figure 3) is dependent on template. However, it has been observed that some other enzymic activity incorporates UTP into an acid-insoluble form in the absence of ATP, GTP, CTP, and functional DNA template. Similar incorporation of UTP into an acid-insoluble form in the absence of the other three ribonucleoside triphosphates and DNA was observed by Reid and Parsons (1971) in the studies of mitochondrial RNA polymerase from rat liver.

The time course of activities of mitochondrial RNA polymerase with both calf thymus DNA and mitochondrial DNA as templates is shown in Figure 4. The reaction showed a rapid incorporation for the first 20 min, which then rapidly diminished. However, higher incorporating activity was observed with mitochondrial DNA as the template.

Sedimentation Properties of the Polymerase. Figure 5 shows the sedimentation pattern of DEAE-Sephadex purified enzyme in sucrose gradients. The sedimentation coefficients of the peaks were measured by comparison to known proteins (see Methods). The first peak has a sedimentation coefficient of 4.3 S and the second peak, presumably an aggregate, has a sedimentation coefficient greater than 11 S. Aggregation of purified mitochondrial RNA polymerase from *Xenopus laevis* and also from *Neurospora crassa* in low-salt glycerol gradients was reported previously (Wu and Dawid, 1972; Kuntzel and Schafer, 1971). Assuming a partial specific volume of 0.73 (globular protein) the 4.3S peak corresponds to a mol wt of 66,500.

Sodium Dodecyl Sulfate Gel Electrophoresis of Mitochondrial RNA Polymerase. Electrophoresis of the DEAE-Sepha-

TABLE III: Antibiotic Sensitivity of Different Peaks Separated on DEAE-Sephadex.

Antibiotic	Inhibition (%) Peak I	Peak II	Peak III	Peak IV
α-Amanitin (40 μg/ml)	82	0	0	0
Rifampicin (10 μg/ml)	0	30	80	14
Actinomycin D (100 μg/ml)	22	0	80	0

TABLE IV: Properties of Mitochondrial RNA Polymerase.

Exptl Conditions	Enzyme Act. (pmol of UMP/mg of Protein per 10 min)	% of Control
Expt 1[a]		
Complete system[c]	130	
$-Mn^{2+}$ (1 mM)	67	52
$-Mg^{2+}$ (4 mM)	60	46
−DNA	13	10
−ATP, GTP, CTP	29	22
+Actinomycin D (100 μg/ml)	86	66
+Rifampicin (20 μg/ml)	10	8
+α-Amanitin (40 μg/ml)	110	85
Expt 2[b]		
Complete system	60	
$-Mn^{2+}$ (1 mM)	31	52
$-Mg^{2+}$ (4 mM) + Mg^{2+} (1 mM)	41	68
−DNA	10	17
−ATP, GTP, CTP	11	18
+Actinomycin D (100 μg/ml)	12	20
+Rifampicin (10 μg/ml)	11	18
+α-Amanitin (40 μg/ml)	96	160

[a] Enzyme fraction from glycerol gradient peak, A_{280} = 0.02 per assay. [b] Enzyme fraction from peak III of DEAE-Sephadex, A_{280} = 0.015 per assay. [c] Complete system is the same as described under Materials and Methods.

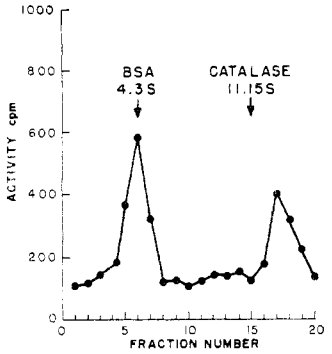

FIGURE 5: Sucrose gradient (5–20% w/v) sedimentation of purified mtRNA polymerase: 0.5-ml fractions (top first) were collected and 100-μl aliquots of each fraction were assayed for RNA polymerase activity Bovine serum albumin and beef liver catalase were run in parallel gradients. Further details are described in the text.

dex purified enzyme preparation showed one major and one minor protein band (Figure 6). The major band accounted for about 70% of the total protein on the gels. The minor protein band migrated slower than the major protein band and accounted for about 30% of the total protein in the gels. The molecular weight of the major protein band is estimated to be 66,000, since it has the same electrophoretic mobility as bovine serum albumin (mol wt 66,000; Low, 1952) in the sodium dodecyl sulfate gels (see Methods). This value is in good agreement with the sedimentation coefficient of 4.3 S of the mitochondrial RNA polymerase. Thus, the membrane-bound mitochondrial RNA polymerase from rat liver appears to be a single polypeptide having a mol wt of 66,000.

Bacterial Contamination. The possibility of bacterial contamination was tested by plating the mitochondrial preparation on nutrient agar and incubating for 24–48 hr at 37°. Colony counts varied between 1.2×10^4 and 1.5×10^4 per mg of mitochondria. The colonies were microscopically diagnosed as staphylococcus. Similar results were obtained by Reid and Parsons (1971). These authors stated that this proportion of bacterial contamination does not interfere with RNA polymerase activity. No bacterial contamination was found in the final enzyme preparation (peak III in Figure 3).

Discussion

Mitochondrial DNA dependent RNA polymerase seems to be tightly bound to the membranes. The data presented here show that at least one of the mitochondrial RNA polymerases has been solubilized from the membranes. The ribonucleoside triphosphate dependent incorporation shown in Table IV is not due to nRNA polymerase contamination. The high dependence of the enzyme activity on the added template as well as its inhibition in the presence of actinomycin D suggest that endogenous DNA has been removed from purified mitochondrial RNA polymerase. The low inhibition of enzyme activity (37%) by actinomycin D, as observed by Reid and Parsons (1971), might indicate the presence of an enzyme–DNA complex. It seems that if the enzyme is associated with endogenous DNA, it may become inaccessible to the inhibitor. It has been observed that prolonged incubation (16 hr) with DNase I, which further removes the endogenous DNA renders the enzyme more susceptible to inhibition by actinomycin D.

Stability of the enzyme decreased as purification proceeded. The crude deoxycholate solubilized enzyme was quite stable

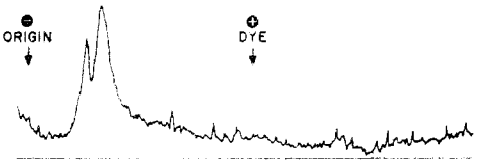

FIGURE 6: Gel electrophoresis of mtRNA polymerase in the presence of sodium dodecyl sulfate. Tracing of the Coomassie Blue stained gel of DEAE-Sephadex purified enzyme (peak III in Figure 3) is shown.

when stored in 20% glycerol at −20°. Similar observations were made in the study of chloroplast RNA polymerase by Bottomley *et al.* (1971). It seems that endogenous DNA might contribute to the stability of the crude enzyme.

Mitochondrial RNA polymerase was distinguished from RNA polymerases residing in the nucleus. Rifampicin has no effect on nRNA polymerases from mammalian cells (Wehrli *et al.*, 1968a,b; Umezawa *et al.*, 1968) including rat liver (Shmerling, 1969). But Reid and Parsons (1971), Shmerling (1969), and Gadaleta *et al.* (1970) have demonstrated rifampicin inhibition of RNA polymerase in the intact mitochondria from rat liver. The previous investigators, however, could not obtain high rifampicin inhibition of the solubilized enzyme due to incomplete purification. It is known that rifampicin inhibits the initiation of RNA synthesis by polymerase. If any endogenous DNA is present in the enzyme, very little rifampicin inhibition would be observed. In our studies, we have demonstrated very high rifampicin inhibition (80%) of the purified mitochondrial RNA polymerase.

The second criterion used to distinguish between the mt- and nRNA polymerases is their respective responses to the specific RNA polymerase inhibitor, α-amanitin. One of the two nuclear polymerases described (Novello and Stirpe, 1970; Kedinger *et al.*, 1970) is highly sensitive to α-amanitin. But α-amanitin has no effect on mtRNA polymerases (Reid and Parsons, 1971; Tsai *et al.*, 1971). In the present studies it has also been found that the purified mtRNA polymerase from rat liver is completely insensitive to α-amanitin.

These differences in the properties of nuclear and mitochondrial RNA polymerases further support the concept that mitochondria may have partial autonomy in biogenesis.

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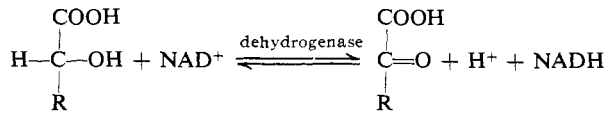
Conformation of Nicotinamide Adenine Dinucleotide Bound to Cytoplasmic Malate Dehydrogenase†

Lawrence E. Webb, Edward J. Hill,‡ and Leonard J. Banaszak*,§

ABSTRACT: The structure of NAD^+ bound to each subunit of cytoplasmic malate dehydrogenase has been determined from an electron density map at 2.5-Å resolution. The structures of the bound coenzyme molecules are closely related by the two-fold rotational symmetry of the dimeric enzyme with the binding sites at the closest point about 20 Å apart. The NAD^+ molecule is in an open conformation with the bases unstacked. The torsion angles are in general agreement with those found in 5′-nucleotides and polynucleotides, with the exception of differences in ribose ring and phosphate orientation, which presumably accommodate binding to the protein. An isolated electron density peak, ascribed to a sulfate ion, is located close to the nicotinamide ring in a position which could be the substrate location during catalysis. The coenzyme structure and binding are very similar to those found crystallographically in lactate dehydrogenase, but some differences are observed. One segment of protein chain, which forms a loop near the coenzyme, is shifted relative to the coenzyme in one of the malate dehydrogenase subunits. This shift differs from the folding down of the same segment observed in the abortive ternary complex of lactate dehydrogenase.

Nicotinamide adenine dinucleotide, NAD^+, along with its 2′-phosphoric acid derivative, $NADP^+$, are coenzymes in a very large number of enzymatic oxidations and reductions. A simple example of this form of metabolic reaction is the oxidation of hydroxy acids



For the enzyme, malate dehydrogenase, R is $\cdot CH_2COOH$, and for lactate dehydrogenase, R is $\cdot CH_3$. In the direction of oxidation, two hydrogen atoms are removed from the carboxylic acid. One hydrogen atom plus one electron are transferred to the 4 position of the nicotinamide ring and one proton is found in the solvent.[1] Figure 1 shows the covalent struc-

† From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110. *Received June 18, 1973.* This work was supported by grants from the National Science Foundation (GB-27437X), National Institutes of Health (GM-13925), and the Life Insurance Medical Research Fund (G-70-26).

‡ Postdoctoral fellow of the National Institutes of Health (1-F02-GM 49896-01). Present address: Department of Physiology, Vanderbilt University Medical School, Nashville, Tenn.

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[1] For a discussion of the chemistry of NAD^+ as related to dehydrogenases, see Bruice and Benkovic (1966).