A DNA LIGASE FROM MITOCHONDRIA OF RAT LIVER

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Received January 20,1976

SUMMARY: An ATP-dependent DNA ligase has been demonstrated in extracts of rat liver mitochondria. The activity may be released from the mitochondria by treatment with hypotonic solutions or a detergent, indicating an intramitochondrial localization. The properties of the partially purified enzyme are similar to those of the nuclear DNA ligase from rat liver.

Several percent of the total DNA of eukaryotic cells is contained within mitochondria. This mitochondrial DNA is often in a closed circular form (1), implying the action of a DNA ligase-like activity for at least one step in its replication. We have found an ATP-dependent DNA ligase in mitochondria isolated from rat liver. This activity is released by osmotic and other treatments in the manner expected for an intramitochondrial localization. The properties of the partially purified enzyme are similar to those of the nuclear DNA ligase from rat liver (2,3,4) but differ from some of those reported for a "soluble" cytoplasmic ligase (4).

MATERIALS AND METHODS:

DNA ligase: DNA ligase and protein were assayed as previously described (3). DNA ligase Fraction IV was isolated from the nuclear fraction of rat liver as before (3).

Mitochondria: Mitochondria were isolated from rat liver by two procedures. All steps were at 0-5°.

Differential centrifugation (5): Liver homogenates were prepared with a Sorvall Omnimixer as before (3) except a medium (Medium H) composed of 0.25 M sucrose--1 mM EDTA (pH 7) (6) was used at 5 ml per g of liver. Immediately before homogenization, 1 volume of 0.07 M phenylmethylsufonyl fluoride (Calbiochem) freshly dissolved in 95% ethanol was added per 35 volumes of Medium H. The homogenate was centrifuged 3 times for 10 min at 600 x g; each time about 3/4 of the supernatant fluid was decanted taking care not to dislodge loosely packed pellets. The final supernatant fluid was centrifuged for 10 min at 6,000 x g and the pellet resuspended in 100 ml of Medium H. This high speed centrifugation was repeated three times more and finally the pellet was weighed, frozen with Dry Ice, and stored at -70°.

Step-wise gradient centrifugation: Mitochondria were prepared by differentia centrifugation as above except the final (unfrozen) high speed pellet from 6 rats was resuspended in 9 ml of Medium H and 3 ml aliquots layered on top of stepwise sucrose gradients composed of 12 ml of 1.0 M sucrose-5 mM EDTA-10 mM Tris Cl (pH 7.6) over 12 ml of 1.5 M sucrose-5 mM EDTA-10 mM Tris Cl (pH 7.6) (8). After centrifugation for 70 min at 20,000 rpm (Spinco, SW 25.1) there was a dense mitocondrial layer at the interface of the 1.0 M and 1.5 M sucrose solutions, and much smaller amounts of material as a pellet and at the interface between the 0.25 M and 1 M layers. The mitochondrial layer was removed with a pipet, centrifuged for 10 min at 6,000 x g, resuspended in 30 ml of Medium H and recentrifuged at 6,000 x g. The final pellet was stored frozen, as above.

Purification of the mitochondrial DNA ligase:

The extraction and G-25 filtration are directly based upon the procedure of Meyer and Simpson (6). All steps were at 0-5°.

Extraction: A partially thawed pellet of mitochondria was ground for 2 min in a mortar (prechilled to -12°) with 2 parts by weight of alumina (A-301, Alcoa) per part of wet weight of pellet. Extraction buffer (0.05 M Tris Cl (pH 8.0)-5 mM β-mercaptoethanol-1 mM EDTA-0.05 M Mg acetate-1 M NaCl) was added (2 ml/g mitochondrial pellet) and grinding continued for 20 sec. The mixture was centrifuged 30 min at 17,000 x g. The pellet was reextracted by briefly stirring with 1/2 the original volume of extraction buffer, recentrifuged, and the supernatant fluids combined (Fraction I, specific activity = 0.3 units/mg, recovery taken as 100%).

Sephadex filtration: Fraction I (27 ml) was desalted by passing through a Sephadex G-25 (Pharmacia) column (3.8 cm^2 x 40 cm, equilibrated and eluted with 25 mM Tris Cl (pH 8.0)-5 mM β-mercaptoethanol-1 mM EDTA). The excluded volume was pooled (Fraction II, specific act. = 0.3, recovery = 90%). Fraction II was frozen in Dry Ice and stored at -70°.

Phosphocellulose chromatography: Fraction II (25 ml) was loaded onto a phosphocellulose column (0.9 cm^2 x 15 cm, equilibrated with 0.01 M K phosphate (pH 6.8)-3 mM β-mercaptoethanol) and eluted with a linear gradient (0 to 0.6 M KCl in the equilibration medium). The only ligase activity detected appeared as a sharp peak at about 0.37 M KCl. Peak fractions were pooled (Fraction III, sp. act. = 16, recovery = 76%). Generally, 1/10 volume of bovine plasma albumin (10 mg/ml) was added and the Fraction III quick-frozen and stored at -70°; this material was dialyzed (2 hrs., vs. 10 mM Tris Cl (pH 8.0)-10 mM β-mercaptoethanol) before use. Recovery of activity upon frozen storage followed by dialysis was 50-70%.

RESULTS AND DISCUSSION:

Mitochondrial isolation and extraction procedures: Extracts of mitochondria contain reproducible levels of ligase irrespective of the exact procedures used to purify the mitochondria or to extract the ligase. For example, mitochondria isolated by different numbers of cycles of differential centrifugation yielded the same ligase specific activity in their extracts as did those mitochondria after a further stepwise gradient centrifugation (Table I). Alumina disruption of mitochondria gave extracts with the most stable ligase activity; however,

TABLE I

DNA ligase activity in extracts prepared from mitochondria isolated by different procedures

Mitochondrial isolation procedures	DNA ligase specific activity
	units/mg protein
Differential centrifugations	
3 low speed + 4 high speed	0.18, 0.22, 0.28
4 low speed + 6 high speed	0.15
Stepwise gradient centrifugation (after 3 low speed + 4 high speed differential centrifugations)	0.22

detergent treatment (0.1% Triton X-100, Calbiochem) and osmotic shock (see below) yielded similar levels of activity.

The latent nature of mitochondrial ligase: Mitochondria rupture when placed in sufficiently hypotonic solutions. This property has been used previously to demonstrate an internal localization of a number of mitochondrial enzymes (9,10,11). We have similarly found that increasing amounts of ligase activity are released into a non-particulate state by exposure to increasingly hypotonic solutions (Fig. 1). The release of ligase is expressed in Figure 1 as a percent of that solubilized by exposure of mitochondria to the detergent, Triton X-100, a treatment often used to release membrane-bounded components (9). The activity released into a non-particulate form before or after osmotic shock was not significantly changed by a subsequent extraction of the particles with 0.2 M NaCl (Fig. 1, squares vs. circles). Such extraction changed the pattern of

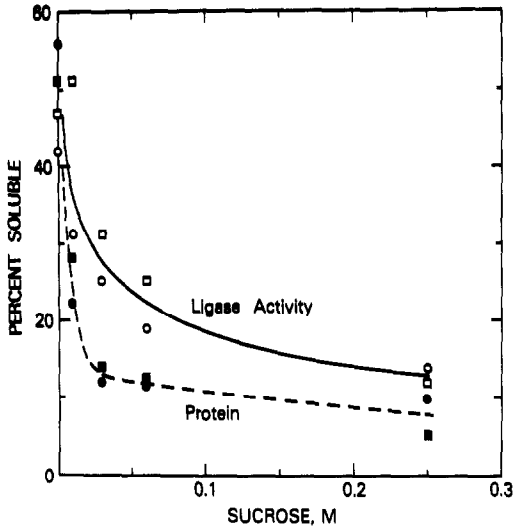

Figure 1. Release of DNA ligase and protein into a soluble form by osmotic treatment of mitochondria. Aliquots of mitochondria (3.0 mg total protein) prepared by differential centrifugation (Methods) were pelleted in two series of tubes. The pellets were resuspended in 0.5 ml of a sucrose solution at the concentration indicated. After 10 min at 0°, one series of tubes (○,●) was brought to 0.2 M NaCl (by addition of 1 M NaCl) while no addition was made to the other series (□ ,■). After an additional 10 min at 0°, the tubes were centrifuged 10 min at 10,000 x g and the supernatant fluids assayed for ligase and protein content. One member of each series was similarly extracted with 0.25 M sucrose containing 0.1% Triton X-100; the data are expressed as percentages of the ligase or protein released by this medium (0.13 or 0.17 ligase unit and 1.0 or 1.1 mg protein, respectively, in the two series).

solubilization of several enzymes when they were adsorbed onto mitochondrial membranes rather than localized internally (11). The similarity between the solubilization of ligase activity and of the bulk protein of the mitochondria (Fig. 1) is again consistent with the internal localization of the enzyme.

Partial purification of the DNA ligase: Ligase was purified ca. 50-fold by gradient chromatography on phosphocellulose. The total yield of activity on such a column was 90-104%; only a single sharp peak of activity was seen except in cases where the protease inhibitor was omitted from the homogenization. The KCl concentration which eluted the activity (0.36-0.38 M) was similar to that which elutes the nuclear enzyme (0.34-0.36 M) (3).

Properties of the mitochondrial ligase: The requirements for mitochondrial ligase activity (Table II) were similar to those of the nuclear enzyme (3). The

TABLE II

Requirements for mitochondrial DNA ligase activity

The complete system contained the usual assay components plus 5 μg of dialyzed ligase Fraction III.

Components	Ligase activity
	pmole/20 min.
Complete	0.029
Omit enzyme	<0.003
Omit $MgCl_2$	<0.003
Omit NaCl	0.004
Omit ATP	<0.003
Omit ATP, add NAD^+ (1 mM) or heated extract of rat liver[a] (0.03 ml)	<0.003
Omit β-mercaptoethanol[b]	0.009
Omit β-mercaptoethanol[b], add p-Chloromercuribenzoate (10 μM)	<0.003
Heated [^{32}P]DNA (10 min, 100°)	<0.003
Heated enzyme (5 min, 65°)	<0.003
Complete[c]	0.037
Add 1 μg of pancreatic DNase	<0.003
Add 1 μg of pancreatic RNase	0.041
Add 1 μg of Pronase	0.035

[a] Extract is supernatant fluid after heating (10 min. 100°) a homogenate of rat liver (1 g per 3 ml H_2O) and centrifuging 10 min at 8,000 x g. An increased blank (0.01 pmole) due to the heated extract has been subtracted.

[b] β-Mercaptoethanol omitted from both dialysis and assay media.

[c] After initial 20 min. incubation with ligase, heat 5 min. 100°; add enzymes indicated and incubate 20 min. 37°.

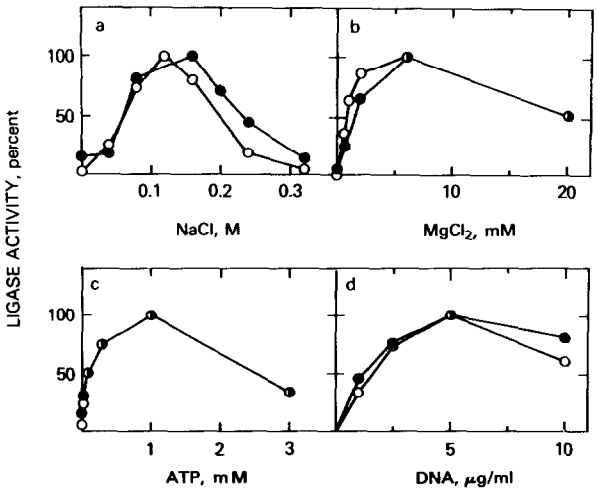

Figure 2. Effects of NaCl, $MgCl_2$, ATP or DNA concentrations upon the rate of mitochondrial and nuclear DNA ligases. Mitochondrial ligase, ●; nuclear ligase, O.

mitochondrial and nuclear ligases responded very similarly to changes in concentration of NaCl, $MgCl_2$, ATP or DNA (Fig. 2). The enzymes had a similar pH optimum (pH 8.0 > pH 7.1 or pH 9.0). Finally, a molecular weight of 10×10^4 was estimated for the mitochondrial ligase (by gel filtration as described in ref. 3, with 5 ml of Fraction II, 83% recovery of activity) which is the same as that previously estimated for the nuclear enzyme (3).

Conclusions:

Previous attempts by us to demonstrate ligase activity in mitochondrial extracts yielded only low levels of very unstable activity ($t_{1/2}$ < 1 day). Two aspects of the present procedure seem to be responsible for obtaining active and fairly stable mitochondrial extracts which yield reproducible chromatographic profiles of ligase activity. First, the alumina extraction technique of Meyer and Simpson (6) was used. Secondly a protease inhibitor was present during the initial cell homogenization.

There are two types of evidence that the ligase activity measured in mitochondrial extracts originates within the mitochondria. First, the level of activity is insensitive to the exact procedure used to purify the mitochondria.

Secondly, the activity is released by exposing the mitochondria to hypotonic media under conditions known to osmotically rupture these organelles.

The mitochondrial ligase is indistinguishable from the nuclear ligase in all the properties tested. We therefore suggest that the same or a closely related enzyme functions in both nuclei and mitochondria from rat liver. In contrast, the "soluble" fraction of the cytoplasm from this tissue has been reported to have one or more ligases (4,12) distinguishable from the nuclear-mitochondrial species by greater molecular weight and somewhat different chromatographic behavior (4). In addition, multiple species of nuclear ligase have been isolated from calf thymus tissue (13).

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