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Intracellular Localization of *Neurospora crassa* Endo-Exonuclease and Its Putative Precursor

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Endo-exonuclease of rapidly growing mycelia of *Neurospora crassa* was found to be distributed in a ratio of about 1.6:1 in vacuoles and in mitochondria where it is associated with the inner membrane. Although the activity in vacuoles was readily released by osmotic shock, very little of that in mitochondria was released by this method. The mitochondrial activity was partially (60 to 70%) released by sonication, and the remaining activity was solubilized in the presence of Triton X-100. An inactive form of endo-exonuclease, activated in vitro by treatment with trypsin, is present in mycelia at a level over four times that of active enzyme. It was found to be distributed in a ratio of about 2.5:1 in the cytosol and in the inner membrane of mitochondria. The mitochondrial protein was more tightly bound than the active enzyme. Very little of the inactive enzyme was released by sonication, but it was solubilized in the presence of Triton X-100. The intracellular distribution of active and inactive forms of endo-exonuclease differs in a mutagen-sensitive mutant of *Neurospora crassa* (*uvs-3*) which shows many pleiotropic effects. The most striking difference in distribution is in the mitochondria where endo-exonuclease is present almost entirely in the inactive form at a level 30% higher than in wild-type mitochondria.

Endo-exonuclease of rapidly growing mycelia of *Neurospora crassa* was first recognized by Fraser et al. (8) as the major nuclease of this organism. This enzyme not only has endonuclease activity with single-stranded DNA (ss-DNA) and RNA, but also has exonuclease activity with double-stranded DNA (ds-DNA). Both activities were later found by Kwong and Fraser (15) to be associated with the same 53-kilodalton polypeptide. They are both dependent on divalent metal ions (Mg^{2+} or Mn^{2+}, but not Ca^{2+} or Zn^{2+}) and both are expressed over the pH range 5 to 9 although in different ratios at different pHs. Chow and Fraser (5) showed that the exonuclease activity of the enzyme is masked in crude extracts of rapidly growing mycelia but can be activated by treating the extracts with trypsin. Thus, sonic extracts of mycelia express a high ss-DNA-specific endonuclease activity. The exonuclease activity of partially purified endo-exonuclease was also shown earlier to be more sensitive to salt than the endonuclease activity as it was preferentially inactivated by endogenous proteases (8). The action of these proteases converted the enzyme to a form that strongly resembled the ss-DNA-specific endonuclease (EC 3.1.30.1) first isolated from stationary-phase starved mycelia by Linn (16).

An inactive form of endo-exonuclease was first purified from *N. crassa* mycelia by Kwong and Fraser (15). Both endo- and exonuclease activities are activated in vitro by treating the protein with trypsin. The protein is hydrophobic and is comprised of a single polypeptide of about 94 kilodaltons. It accumulates in high amounts in mycelia during rapid growth and disappears in stationary phase (5). Although there is no direct evidence as yet that this protein is related at the molecular level to the smaller active endo-exonuclease, the enzymological properties of the trypsin-activated protein so closely resemble those of the active enzyme that the inactive protein is tentatively assumed to be a precursor of the active endo-exonuclease (15).

The biological role of *N. crassa* endo-exonuclease is not known, but the observations (5, 7) that there are partial deficiencies in the levels of active enzyme in some mutagen-sensitive mutants suggest a possible role in DNA repair. It was therefore of interest to examine the intracellular localizations of this enzyme and its putative precursor to determine, in particular, whether or not the enzyme is present in DNA-containing organelles and to compare these localizations with those in a nuclease-deficient mutagen-sensitive mutant.

MATERIALS AND METHODS

Strains, media, and growth conditions. Wild-type *N. crassa* 74-OR23-1VA was used in this work; *uvs-3a*

was a well-backcrossed strain obtained from E. Käfer (11). Conidia were grown on 10 ml of enriched solid agar (Neurospora culture agar; BBL Microbiology Systems, Cockeysville, Md.) for 5 to 7 days at room temperature in inverted 125-ml Erlenmeyer flasks. Conidia from one agar culture were used to inoculate two 250-ml flasks containing 100 ml of Neurospora minimal medium (Difco Laboratories, Detroit, Mich.) with 2% sucrose as carbon source. Mycelia were grown for 16 h at 30°C in an incubator (New Brunswick Scientific Co., New Brunswick, N.J.) shaking at a rate of 150 rpm. The average yield of mycelia was 1.8 g (wet weight) per flask.

Extraction of mycelia. For preparation of nuclei, mycelial mats (30 to 40 g) were washed with water and cut into small pieces. They were blended at 0 to 8°C in a Waring blender at top speed for three 30-s bursts with 4 g of glass beads (0.17 to 0.18-mm diameter; Sargent-Welch Scientific Co., Toronto, Ontario, Canada) and 2.5 ml of extraction medium per g of mycelia. The extraction medium contained 1.0 M sorbitol, 5% Ficoll, 20% glycerol, 5 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.5% Triton X-100, a minor modification of buffer A of Hautala et al. (10). The glass beads were washed once with the same medium to give a combined volume of original extract and wash of 7 to 8 ml/g of mycelia. For preparation of all other cell fractions, the same method of extraction was used, except that the extraction medium was 20% sucrose–1 mM EDTA (sucrose-EDTA) (22).

Preparation of cell fractions. Crude nuclear pellets were prepared from the extract by the method of Hautala et al. (10), resuspended in 2.5 ml of the detergent-containing extraction medium per g of mycelia (see above), and filtered through glass wool to remove pieces of unbroken mycelia. The nuclei were then resedimented and resuspended in 1 ml of buffer B of Hautala et al. (10), which does not contain Triton X-100, per g of mycelia. Samples of the suspension (6 ml) were layered on linear 40 to 90% gradients (30 ml) of Percoll in 0.25 M sucrose and centrifuged at 10,000 × *g* for 1 h in a refrigerated centrifuge (RC-2B; Ivan Sorvall, Inc., Norwalk, Conn.). Visible bands in the regions of 60 to 70% Percoll were collected, pooled, and sedimented at 9,000 × *g* for 40 min to recover the purified nuclei. The overall yield of DNA from the mycelia was 42%, and the ratio of DNA to RNA to protein was 1:2.7:6.2, comparable to that obtained previously (10). In other experiments, crude nuclei were washed once with buffer B of Hautala et al. (10) and purified by the method of Krumlauf and Marzluf (14) rather than on Percoll gradients. This method involves the use of a buffer containing 0.15 M KCl for the purpose of reducing nonspecific adsorption of nuclease released by detergent action from other organelles (see below).

Heavy vacuoles were prepared from mycelial extracts in sucrose-EDTA by centrifuging extracts at 500 × *g* for 10 min to remove unbroken cells and debris and then recentrifuging the supernatant at 5,000 × *g* for 10 min (22). The pellets were washed once with sucrose-EDTA. A mixture of light vacuoles and mitochondria was sedimented from the 5,000 × *g* supernatant by centrifuging this fraction at 25,000 × *g* for 40 min. The 25,000 × *g* pellets were washed once with sucrose-EDTA and resuspended in the same medium. For preparation of light vacuoles and purified mitochondria, 5-ml samples were layered on linear 35 to 60% sucrose gradients (8 ml). In other cases (e.g., see Fig. 1), 2-ml samples were layered on the same gradients, but the gradients were supported on 3-ml 90% sucrose cushions. The light vacuoles and mitochondria were separated by centrifuging the gradients at 135,000 × *g* for 90 min in an SW-40 rotor in an ultracentrifuge (L2-65B; Beckman Instruments, Inc., Fullerton, Calif.). Under these conditions, the mitochondria banded in the middle of the gradients, whereas the vacuoles sedimented either to the bottoms of the tubes (preparative gradients) or to the tops and through the 90% sucrose cushions. Appropriate fractions were collected from the tops of the gradients, pooled, diluted with sucrose-EDTA, and centrifuged at 25,000 × *g* for 40 min to recover the purified organelles.

The microsomes and cytosol fractions were prepared from the 25,000 × *g* supernatant by centrifuging at 135,000 × *g* for 90 min in the ultracentrifuge described above. The 135,000 × *g* supernatants (cytosol fraction) were decanted from the pellets (microsomes).

Submitochondrial localization. The submitochondrial localizations of endo-exonuclease and its putative precursor were carried out by the digitonin titration method originally described by Schnaitman and Greenawalt (27) and modified for *N. crassa* mitochondria by Cassady et al. (4). Solubilization of the following mitochondrial marker enzymes was also followed: an intermembrane space marker, adenylate kinase (AK) (EC 2.7.4.3); an inner membrane marker, succinate dehydrogenase (SDH) (EC 1.3.99.1); and a matrix marker, glutamate dehydrogenase (GDH) (EC 1.4.1.4). Purified mitochondria from sucrose gradients (see above) were used for these experiments. Inner and outer mitochondrial membrane fractions were also prepared from digitonin-treated mitochondria by discontinuous sucrose density gradient fractionation as described by Cassady et al. (4).

Electron microscopy. The purity of the two types of organelles found to contain endo-exonuclease, vacuoles and mitochondria, was examined by electron microscopy. Suspensions of organelles in sucrose-EDTA (1.0 ml) were overlaid with 1 ml of 5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.3) and allowed to stand for 15 min at room temperature. Then the two layers were mixed and allowed to stand a further 15 min, after which the mixture was centrifuged at 25,000 × *g* for 10 min. The supernatants were allowed to stand overnight in contact with the pellets. The next day the pellets were washed twice with 0.1 M sodium cacodylate buffer containing 7% sucrose and, after resedimentation, postfixed in a mixture containing 1.5% potassium ferrocyanide and 1% osmium tetroxide (M. J. Karnovsky, Abstr. Am. Soc. Cell Biol. 1971, p. 146) for 2 h. The samples were dehydrated in turn with 70 and 100% ethanol and then with propylene oxide. They were sectioned to approximately 70 to 80 nm with an Ultratome III (LKB Instruments, Inc., Rockville, Md.) and embedded in Epon 812. Sections were stained on the electron microscope grids successively with 2% aqueous uranyl acetate and lead citrate (26). The electron micrographs were taken with a Philips 300 electron microscope.

Determinations of DNA, RNA, and protein. DNA was determined by the method of Giles and Myers (9), RNA was determined by the method of Schneider (28),

and protein was determined by the method of Lowry et al. (19) using bovine serum albumin as a standard.

Enzyme assays. Endo-exonuclease was routinely assayed at 37°C by measuring the Mg^{2+}-dependent (10 mM) release of perchloric acid-soluble material absorbing at 260 nm from ss-DNA (initial concentration, 0.67 mg/ml) at pH 8.0 by the method of Fraser (7). The putative endo-exonuclease precursor was assayed by the same method after activation of the protein by treatment with trypsin (EC 3.4.21.4) as described previously by Kwong and Fraser (15). Crude fractions containing a high concentration of protein required pretreatment with trypsin (100 to 200 μg/ml) for 30 min at 37°C, whereas fractions containing less protein required pretreatment with only 2 to 20 μg of trypsin per ml. In each case, the reaction was stopped effectively by the addition of a twofold excess of soybean trypsin inhibitor. The difference in ss-DNase activity measured with and without trypsin activation was taken as a measure of the amount of precursor. The presence of digitonin or up to 2% Triton X-100 did not interfere with the activation. One unit of enzyme activity is defined as that amount of enzyme which releases 1.0 U (of absorbance at 260 nm) of acid-soluble material in 30 min under the conditions of assay. This is equivalent to the release of 65 nmol of nucleotides per min.

Several vacuolar marker enzymes were assayed. Two proteinase activities were measured by the methods of Siepen et al. (29), carboxypeptidase activity with benzoyltyrosine-*p*-nitroanilide (Bz-TYR-Nanase) in 0.1 M Tris-hydrochloride (pH 7.5) and acid proteinase activity with denatured hemoglobin in 0.1 M acetate buffer (pH 4.2) in the absence or presence of 10 μg of the acid-proteinase inhibitor, pepstatin, per ml. The carboxypeptidase and pepstatin-sensitive acid proteinase activities correspond, respectively (29), to proteinases C and A of yeasts (EC 3.4.16.1 and EC 3.4.23.6). Another proteinase not localized in vacuoles, but in the cytosol, aminopeptidase activity with lysine *p*-nitroanilide (LYS-Nanase; EC 3.4.11.6), was also assayed by the method of Siepen et al. (29). The positive vacuolar markers, acid phosphatase (EC 3.1.3.2) and α-mannosidase (EC 3.2.1.24), were assayed by methods described previously by Lowry (18) and by Vaughn and Davis (31), respectively. The mitochondrial marker enzymes (see above) AK, SDH, and GDH were assayed by the methods of Peeters-Joris et al. (25), King (12), and Coddington and Fincham (6), respectively. Membrane-bound ATPases (EC 3.6.1.3) were assayed in the absence and presence of inhibitors (Na_3VO_4, NaN_3, and *N*,*N*′-dicyclohexylcarbodiimide) by the procedures of Bowman and Bowman (3). Inorganic phosphate was determined by the method of Stanton (30).

Chromatography. Sonic extracts of washed heavy vacuoles, washed 25,000 × *g* pellets (containing both light vacuoles and mitochondria), and purified mitochondria from sucrose gradients in 20 mM Tris-hydrochloride–1 mM EDTA (Tris-EDTA) (pH 7.5) were chromatographed on DEAE-Sepharose by the method described by Chow and Fraser (5). The fractions eluted from DEAE-Sepharose containing endo-exonuclease activity were pooled and dialyzed overnight against 20 mM Tris-hydrochloride–5 mM EDTA (pH 7.5) and chromatographed on ss-DNA–cellulose by the method of Alberts and Herrick (1). Digests of ss- and ds-DNA by endo-exonuclease purified from organelles as described above were chromatographed on small Sephadex G-100 columns as described previously by Birnboim (2) to determine qualitatively the mode of DNA degradation (endo- or exonucleolytic). The degradations were carried out in the presence of 10 mM Mg^{2+} and halted by the addition of 20 mM EDTA. [^{3}H]dCMP was added to the digests as a marker mononucleotide.

Chemicals. Calf thymus DNA (type I), bovine trypsin (type III), soybean trypsin inhibitor, pepstatin, phenylmethylsulfonyl fluoride (PMSF), and *p*-nitrophenyl phosphate were purchased from the Sigma Chemical Co., St. Louis, Mo.; yeast phenylalanine tRNA was from Boehringer-Mannheim Corp., New York, N.Y.; benzoyltyrosine and lysine *p*-nitroanilides were from Serva Feinbiochemica, Heidelberg, West Germany; and Percoll was from Pharmacia Fine Chemicals, Piscataway, N.J. [^{3}H]dCMP was obtained from New England Nuclear Corp., Boston, Mass. Membrane-bound ATPase inhibitors (see above) were obtained from R. J. Poole, Department of Biology, McGill University, Montreal, Quebec, Canada. The sources of all other chemicals and materials have been described previously (5, 7, 8).

RESULTS

Nuclei. Nuclei isolated by the method of Hautala et al. (10) and extracted by sonication in Tris-EDTA (pH 7.5) were found to contain only 2% of the total active endo-exonuclease found in mycelia and even less (0.6%) of the total trypsin-activatable (inactive) endo-exonuclease activity. The level of active enzyme seen here may be artificially high owing to adsorption of enzyme released from other organelles (see below) by the Triton X-100 used in the extraction medium, since washing the nuclei with buffers of high osmolality containing salt reduced the endo-exonuclease content by 85 to 95%, i.e., to levels of around 30 to 40 U/mg of DNA. This reduction was observed upon washing with buffer B of Hautala et al. (10) containing 0.15 M KCl or with the buffer used by Krumlauf and Marzluf (14) to purify nuclei which contains 0.1 M KCl. Neither of these washes altered the microscopic appearance of the nuclei as examined by the method of Hautala et al. (10), but some breakage of nuclei was observed to occur during the salt washes.

Vacuoles. Preliminary studies showed that when a crude extract of young mycelia in sucrose-EDTA (500 × *g* supernatant) was centrifuged in linear sucrose gradients, the endo-exonuclease activity was separated into two major fractions, a vacuolar fraction sedimenting through the gradients with no associated SDH activity and a fraction cobanding with SDH activity (mitochondria). Differential centrifugation (see above) of this crude extract resulted in sedimentation of 15% (650 U/10 g of mycelia) of the endo-exonuclease activity in heavy vacuoles at 5,000 × *g* without detectable SDH activity. When the washed 5,000 × *g* pellet was centri-

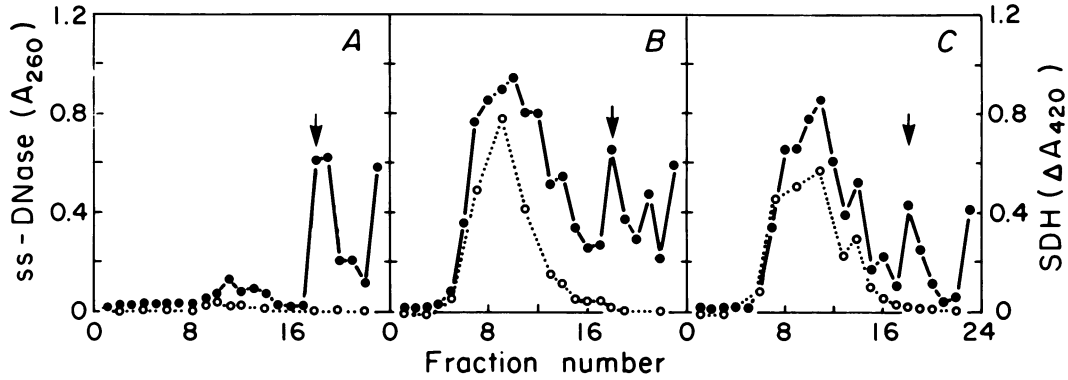

FIG. 1. Centrifugation of heavy vacuoles (washed 5,000 × *g* pellet) and washed 25,000 × *g* pellet on linear 35 to 60% sucrose gradients. The washed pellets were suspended in sucrose-EDTA and layered on gradients supported by 90% sucrose cushions. Sedimentation was from left to right at 135,000 × *g*. Each gradient was analyzed for ss-DNase activity (●) and SDH activity (○). (A) Washed 5,000 × *g* pellet centrifuged for 30 min; (B) washed 25,000 × *g* pellet centrifuged for 30 min; (C) as in (B), but centrifuged for 90 min. The arrow on each panel indicates the top of the 90% sucrose cushion. A_{260}, Absorbance at 260 nm; A_{420}, absorbance at 420 nm.

fuged in a sucrose gradient, the endo-exonuclease activity was found (Fig. 1A) to sediment to the top (indicated in Fig. 1A by an arrow) and through the 90% sucrose cushion. About 75% of the endo-exonuclease activity in crude extracts sedimented at 25,000 × *g* along with most of the SDH activity (see below), but the 25,000 × *g* pellets also contained vacuoles which could be cleanly separated from the mitochondria in sucrose density gradients provided that there was sufficient centrifugation time (90 min) (Fig. 1B and C). The light vacuoles recovered from the bottom of the gradient were also free of detectable SDH activity but contained about the same amount of endo-exonuclease activity (1,700 U/10 g of mycelia) as found in the mitochondria recovered from the gradients. Not all of the vacuolar activity is seen in the gradient profiles (Fig. 1A and C) because a large fraction of it was recovered from material which had pelleted on the bottoms of the centrifuge tubes.

Many of the properties of the heavy and light vacuoles purified from wild-type mycelia were qualitatively the same. Both preparations were osmotically stable. No endo-exonuclease activity was released, even upon storage overnight, if the particles were maintained in sucrose-EDTA. However, 96% of the activity was released in each case by osmotic shock if the particles were suspended in 1 ml of Tris-EDTA (pH 7.5) per g of mycelia. After recentrifuging the osmotically shocked suspensions for 1 h at 100,000 × *g* (3) and washing the pellet (membrane) fraction in Tris-EDTA containing 0.1 M KCl, we recovered only 4% of the total endo-exonuclease activity in the salt-washed membrane fraction. The membrane fractions derived from the heavy and light vacuoles contained, respectively, 73 and 56% of the total protein. When they were dissolved in Tris-EDTA containing 2% Triton X-100, a threefold increase in endo-exonuclease activity was observed. These results indicate that at most 11% of the total vacuolar endo-exonuclease activity was associated with the membrane fractions. The membrane fraction, but not the 100,000 × *g* supernatant, also contained *N,N'*-dicyclohexylcarbodiimide-sensitive ATPase activity. The ATPase activity also showed additive inhibitions (18 and 13%, respectively) with 20 μM Na_3VO_4 and 5 mM NaN_3. These results indicate the presence of slight contaminations with plasma and mitochondrial membranes (3). Thus, it is not possible to conclude from these observations which type of membranes have the small amount of associated endo-exonuclease activity, but the activity is most likely associated with the contaminating mitochondrial membranes (see below). No trypsin-activatable endo-exonuclease activity was detected in extracts of purified vacuoles prepared by osmotic shock, by sonication, or by treatment with 2% Triton X-100 or in membrane fractions derived from the vacuoles and dissolved in 2% Triton X-100. Furthermore, fresh extracts of vacuoles made in the absence and presence of a battery of protease inhibitors (1 mM PMSF, 1 μg of pepstatin per ml, 1 mM EDTA) did not show any differences in the levels of expressed endo-exonuclease activities. The activity (ss-DNase only measured) in extracts, even in the absence of protease inhibitors, was stable in the cold over a 24-h period.

Endo-exonuclease was present in the heavy and light vacuoles in a ratio of about 1:2.6. The same distribution was also found for a number of vacuolar marker enzymes (Bz-TYR-Nanase,

acid protease, alkaline and acid phosphatases, and α-mannosidase). Both heavy and light vacuoles also contained glycosidase activity at pH 4.6 with the β-*p*-nitrophenyl glycoside of *N*-acetylglucosamine, but this was not quantitated. Less than 0.2% of the total LYS-Nanase, a cytosolic marker, was present in the vacuole preparations.

Electron microscopic examination of the heavy and light vacuole preparations (Fig. 2A and C) showed that they both contained a large number of membrane-limited electron-dense particles with no visible contamination by intact mitochondria (compare Fig. 2B), although contamination by other material is evident. Some of these particles had apparently lost portions of their electron-dense material during preparation and, where this occurred, disruptions in the membranes can be seen. The electron-dense material in the vacuoles may be polyphosphates (31). When the vacuoles were osmotically shocked and the resulting lysates were centrifuged at high speed, the membranes and most of the contaminating material (protein) sedimented, unlike most of the endo-exonuclease (see above).

Mitochondria. About 85% of the SDH activity was recovered in the 25,000 × *g* pellets (see above), but variable amounts (5 to 20%) were also recovered in the 25,000 × *g* supernatant. Since the recoveries of SDH and endo-exonuclease activities in the 25,000 × *g* supernatant increased when the time of blending the mycelia with glass beads was increased, the variability in recoveries is attributed mainly to variability in disruption of mitochondria. The mitochondria in the 25,000 × *g* pellet formed a rather broad band in the sucrose density gradients, as judged from the banding of SDH activity (Fig. 1B and C). After only 30 min of centrifugation, the ratio of endo-exonuclease to SDH activities on the leading edge of the band was higher than in other fractions containing SDH activity (Fig. 1B), but after 90 min of centrifugation (Fig. 1C), the two activities banded in the middle of the gradient in approximately the same ratio. In this case, the mitochondria recovered from the gradients, although they contained about 50% of the endo-exonuclease in the 25,000 × *g* pellet, were nearly free of vacuoles, as determined by electron microscopy (Fig. 2B) and by assay of vacuolar marker enzymes after suspending the mitochondria in Tris-EDTA without sonication (osmotic shock) or after sonication (3 to 6 min) in the same medium. Less than 10% of the acid protease and α-mannosidase activities and 10% of the total Bz-TYR-Nanase activity (0.014 U/10 g) found in mycelia were present in the purified mitochondria. In addition, osmotic shock, which released 96% of the endo-exonuclease activity from vacuoles, released less than 10% of the endo-exonuclease activity from purified mitochondria. The mitochondrial preparation also contained only 6% of the cytosolic marker (LYS-Nanase) activity (0.064 U/10 g of mycelia).

Endo-exonuclease was partially (80%) released from mitochondria approximately in parallel with DNA by sonication, and the remaining 20% of endo-exonuclease activity and DNA was solubilized by treatment with Triton X-100. The total yield of endo-exonuclease activity varied from one experiment to the next unless the mitochondria were isolated in the presence of 1 mM PMSF. In this case, the yield was 1,500 U/10 g of mycelia. After standing overnight in sucrose-EDTA or after being stored frozen, the recovery of active enzyme in sonic extracts made in the absence of PMSF was often over three times that obtained from freshly prepared mitochondria. This proved to be due to the presence of a large amount of inactive endo-exonuclease which was activated upon storage. This trypsin-activatable activity was only slightly (20%) released by the most vigorous sonication, but was solubilized completely in the presence of Triton X-100. The total activity in Triton-solubilized extracts of wild-type mitochondria after trypsin activation was always about 7,000 U/10 g of mycelia, of which about 80% was trypsin-activatable activity.

Submitochondrial localization. The results of experiments to determine submitochondrial localization of the active endo-exonuclease are summarized in Table 1. The extractions with different amounts of digitonin were carried out in the absence or presence of 0.15 M KCl to reduce possible nonspecific adsorption of the enzyme on mitochondrial membranes. The results in both cases were found to be qualitatively the same. Significant amounts of ss-DNase activity were solubilized only at digitonin concentrations of 0.8 mg/mg of mitochondrial protein and above. At these concentrations, the inner membrane marker (SDH) was beginning to be solubilized as well, whereas the matrix marker (GDH) was completely released at 0.4 to 0.6 mg of digitonin per mg of protein, and the intermembrane-space marker (AK) was significantly solubilized even at 0.2 to 0.4 mg/mg of protein. These results provide evidence that the mitochondrial ss-DNase activity is associated with the inner membrane. A separate experiment showed that inactive endo-exonuclease was also not solubilized appreciably until the digitonin concentration was 0.8 mg/mg of protein or higher (data not shown).

Further evidence that active endo-exonuclease is associated with the inner membrane of mitochondria was obtained by sedimenting digi-

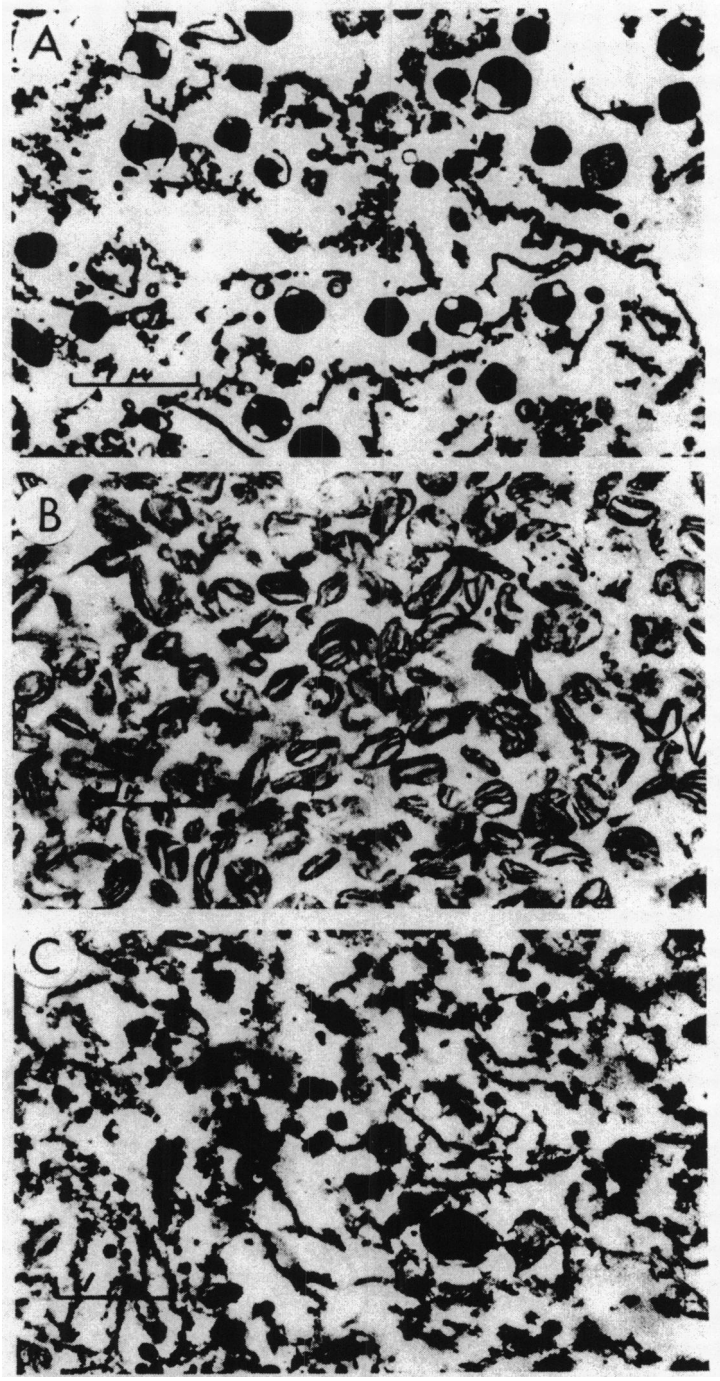

FIG. 2. Electron micrographs of subcellular fractions of young mycelia. The bars (1 μm) on each panel indicate the sizes of the structures examined. (A) Heavy vacuole fraction (5,000 × *g* pellet) after centrifugation for 30 min in a 35 to 60% linear sucrose gradient as described for Fig. 1A (fractions 18–20); (B) purified mitochondria after centrifugation for 90 min in a 35 to 60% sucrose gradient as for Fig. 1C (fractions 7–12); (C) light vacuoles from the bottom of a 35 to 60% sucrose gradient centrifuged for 90 min as for Fig. 1C, but without the 90% sucrose cushion.

TABLE 1. Endo-exonuclease and marker enzyme activities released from purified mitochondria with increasing amounts of digitonin[a]

Enzyme assayed	No. of expts	0.15 M KCl	% of total enzyme activity with the following amt of digitonin (mg/mg of protein)					
			0	0.2	0.4	0.6	0.8	1.0
ss-DNase	5	−	0	0.8	2.0	5.9	13	16
	3	+	0	1.2	4.8	11	17	16
AK	3	−	0	11	42	35	65	48
	3	+	0	28	56	65	84	84
GDH	1	−	0	43	82	82	100	100
	1	+	0	53	65	85	100	100
SDH	3	−	0	0	0	2.7	5.0	7.3
	3	+	0	0.5	2.1	6.8	9.1	8.3

[a] Purified mitochondria were prepared as described for Fig. 1C and suspended in 0.25 M sucrose–0.15% bovine serum albumin in the absence (−) or presence (+) of 0.15 M KCl to a final concentration of about 10 mg of mitochondrial protein per ml. Samples containing a known amount of protein were treated with 0 to 1.0 mg of digitonin per mg of protein for 20 min on ice and then centrifuged at 25,000 × *g* for 30 min. The supernatants were assayed for endo-exonuclease, AK, GDH, and SDH. Activities are expressed as percentage of totals measured after solubilization in 2% Triton X-100. The results from several experiments are averaged.

tonin-treated mitochondria (0.45 mg of digitonin per mg of mitochondrial protein) in step sucrose gradients by the method of Cassady et al. (4) in the absence or presence of 0.15 M KCl (Fig. 3A and B, respectively). Most of the matrix marker enzyme (GDH) was found in the soluble fraction on top of the 1.0 M sucrose with only a small amount sedimenting on top of the 1.9 M sucrose layer. A significant amount of the intermembrane-space marker (AK) was also solubilized, but almost no endo-exonuclease or SDH was solubilized either in the presence or in the absence of KCl. The latter activities remained on top of the 1.9 M sucrose layer. In this experiment, the recoveries of total activities from the gradient were 90, 71, 64, and 42%, respectively, for endo-exonuclease, SDH, AK, and GDH.

Microsomes and cytosol. Only about 10% of the total active endo-exonuclease activity in extracts of mycelia in sucrose-EDTA remained in the 25,000 × *g* supernatant. The activity was Mg^{2+}- but not Ca^{2+}-dependent (see below). About 40% of it sedimented at 135,000 × *g* with the microsome pellet. On the other hand, a very high amount of trypsin-activatable endo-exonuclease activity was present in the 25,000 × *g* supernatant. The activation of ss-DNase activity with trypsin in this fraction was 40- to 50-fold, yielding 13,600 U of trypsin-activatable activity per mg of mycelia. Of this activity, 30% sedimented with the microsomes at 135,000 × *g*. It was completely trypsin sensitive since no further increase was observed if the microsomes were solubilized in 1% Triton X-100.

Comparison of distribution of endo-exonuclease in wild type and *uvs-3* mutant. The quantitative distribution of active and trypsin-activatable endo-exonuclease is summarized in Table 2 and compared with that found by the same methods in the mutagen-sensitive *N. crassa uvs-3* mutant, in which a partial deficiency of active enzyme was previously observed in crude sonic extracts of mycelia (5). It is clear that the endo-

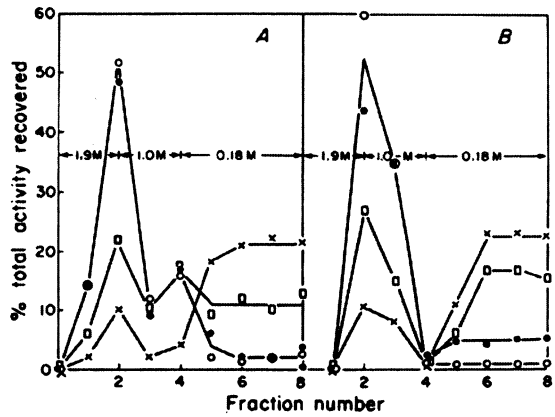

FIG. 3. Step sucrose gradient of purified mitochondria treated with digitonin. Purified mitochondria were obtained as described for Fig. 1C and treated with 0.45 mg of digitonin per mg of mitochondrial protein in the absence (A) or presence (B) of 0.15 M KCl. The mitochondria in 0.18 M sucrose were layered on top of a two-step gradient consisting of 1.9 M sucrose (bottom layer) and 1.0 M sucrose (top layer). Sedimentation was from right to left at 39,000 × *g* for 1 h. Symbols: ●, ss-DNase; ○, SDH; □, AK; x, GDH.

TABLE 2. Intracellular distribution of active and inactive endo-exonuclease in rapidly growing mycelia of wild-type *N. crassa* and the mutagen-sensitive *uvs-3* mutant[a]

Cell fraction	Wild type		*uvs-3*	
	Active enzyme (U/10 g)	Inactive enzyme (U/10 g)	Active enzyme (U/10 g)	Inactive enzyme (U/10 g)
Nuclei	100	110	30	40
Vacuoles	2,350	0	1,480	0
Mitochondria	1,500	5,400	160	7,000
Microsomes	190	4,100	30	4,000
Cytosol	280	9,500	60	9,300

[a] The levels of each form of enzyme are given in units per 10 g of mycelia (fresh weight) and are a compilation from three types of experiments as described in the text, one to determine the levels in nuclei (no salt wash), one to determine the levels in purified mitochondria (in the presence of protease inhibitors to inhibit activation of inactive endo-exonuclease), and a third to determine the levels in other cell fractions.

exonuclease activity of the wild type is present mainly in two different types of organelles, vacuoles and mitochondria, in a ratio of about 1.6:1. In *uvs-3*, there appears to be a partial deficiency of active endo-exonuclease in all cell fractions examined, but the most striking difference is an almost complete deficiency of active enzyme in the mitochondria. The inactive endo-exonuclease, on the other hand, is mainly in the cytosol and microsomes in both the wild type and the mutant, but is also present in mitochondria. The ratio is about 2.5:1. The level of inactive endo-exonuclease in *uvs-3* mitochondria is about 30% higher than in wild-type mitochondria (see below). It should be noted that Table 2 does not include all cell organelles. It is possible that minor amounts of active or inactive endo-exonuclease could be associated with the vacuolar membrane, plasma membrane, peroxisomes, or other organelles. In wild-type *N. crassa*, the vacuoles and mitochondria contain at least 87% of the active endo-exonuclease found in crude extracts, whereas the cytosol, microsomes, and mitochondrial fractions contain 99% of the total detectable inactive endo-exonuclease.

Comparison of properties of vacuolar and mitochondrial endo-exonucleases. Although the same assay has been used to monitor endo-exonuclease activity in different cell fractions throughout this work, the assay is not very specific in principle. It measures all Mg^{2+}-dependent ss-DNase activity at pH 8.0, whether due to endo-exonuclease or not. The following observations, however, indicate that most of the activity is indeed associated with endo-exonuclease. When sonic extracts in Tris-EDTA of heavy vacuoles, 25,000 × *g* pellets (mixture of light vacuoles and mitochondria), or purified mitochondria were chromatographed on DEAE-Sepharose equilibrated with Tris-EDTA, over 90% of the ss-DNase activity adsorbed, and it was subsequently eluted with a linear 0 to 0.5 M salt gradient in the range of 0.10 to 0.25 M NaCl. All of the heavy vacuolar and 25,000 × *g* pellet activity recovered from DEAE-Sepharose then bound to ss-DNA–cellulose and, in each case, was eluted in 90% yield as a single component in a linear 0 to 1.0 M salt gradient in the range of 0.50 to 0.75 M NaCl (observation of T. Y.-K. Chow in this laboratory). For the 25,000 × *g* pellet activity, the overall yield from the original extract was greater than 80%, and the recovery of protein from the ss-DNA–cellulose step was so small that a purification of at least 1,500-fold from the extract is indicated. Similar purifications are indicated for ss-DNase activity from extracts of the large vacuoles and purified mitochondria, although the overall recoveries of activity are somewhat lower, both being in the neighborhood of 30% (details to be presented at a later date). The tentative conclusion is that the chromatographic properties of the ss-DNase activities from the three different organelle preparations are very similar.

When digests of ss-DNA and ds-DNA in the presence of 10 mM Mg^{2+} by either the heavy vacuolar, 25,000 × *g* pellet, or mitochondrial enzymes recovered from ss-DNA–cellulose were examined by chromatography on small Sephadex G-100 columns by the method of Birnboim (2), the profiles were qualitatively the same as those seen previously for purified *N. crassa* endo-exonuclease (8). The data are shown for the heavy vacuole enzyme. With ss-DNA (Fig. 4A), as the time of digestion progressed from 0 to 30 min, the level of oligonucleotides increased, as can be seen by substantial increases in material in fractions 6 through 9 eluting between the excluded high-molecular-weight DNA (open circles) and the marker mononucleotide (indicated by arrow). On the other hand, when ds-DNA was used as the substrate (Fig. 4B), the profiles showed no such increases in material eluting between high-molecular-weight DNA and the marker mononucleotide. Instead, as the amount of excluded DNA decreased, the amount of material eluting in the region of the mononucleotide increased. The activity with ss-DNA is thus qualitatively endonucleolytic, whereas that with ds-DNA is exonucleolytic in character. The ss-DNA-binding enzymes from large vacuoles, the 25,000 × *g* pellet, and purified mitochondria also had Mg^{2+}-dependent endonucleolytic activity with RNA (data not shown). This was expressed optimally with tRNA as the substrate in the presence of

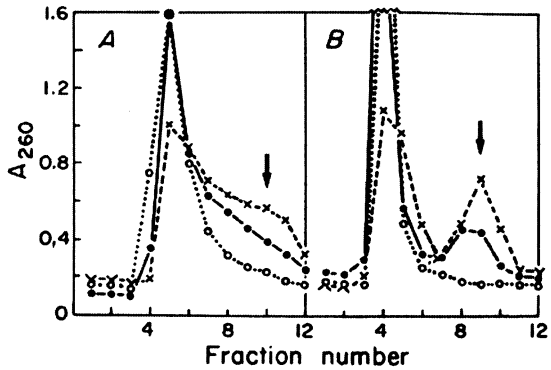

FIG. 4. Gel filtration through Sephadex G-100 of digests of ss-DNA and ds-DNA with ss-DNA-binding nuclease derived from heavy vacuoles. Incubation mixtures (0.6 ml) contained 0.67 mg of DNA per ml, 0.1 M Tris-hydrochloride (pH 8.0), 10 mM Mg^{2+}, and either 3.8 U of enzyme (ss-DNA) or 19 U of enzyme (ds-DNA) per ml. Incubations were carried out at 37°C. Samples (200 μl) were removed at 0, 15, and 30 min and mixed with 20 μl of 0.2 M EDTA to stop the reaction. Each digest was loaded on a Sephadex G-100 column (0.6 by 29 cm) equilibrated and developed with 0.1 M Tris-hydrochloride (pH 7.5) containing 10 mM EDTA. The absorbance at 260 nm (A_{260}) of each fraction was determined. The arrow on each panel indicates the position of elution of the marker mononucleotide ([^{3}H]dCMP). (A) ss-DNA; (B) ds-DNA. Symbols: O, Zero time control; ●, 15-min digest; x, 30-min digest.

0.1 to 0.2 mM Mg^{2+} in excess of EDTA (present in the purified enzymes). Both ss- and ds-DNase activities of the three enzyme preparations were supported by Mn^{2+} as well as Mg^{2+}, but neither activity was seen with Ca^{2+} or Zn^{2+}. In 0.1 to 0.2 mM Mg^{2+}, only endonuclease activity was detected with ss-DNA. Under these conditions, no ds-DNase activity was expressed. Optimal ds-DNase (exonuclease) activity occurred with 10 mM Mg^{2+}. Thus, the enzymological properties of the vacuolar and mitochondrial enzymes are also very similar.

DISCUSSION

Although a vacuolar nuclease in *N. crassa* has not been previously described, Linn and Lehman have reported (17) the localization of a nonstrand-specific endonuclease in mitochondria from mycelia grown well into stationary phase. Martin and Wagner (21) showed that apparently the same enzyme was associated with the mitochondrial inner membrane. The mitochondrial endo-exonuclease examined in the present work has been purified from rapidly growing mycelia. Despite some similarities to the endonuclease described previously (17), especially in divalent metal ion requirements and endonucleolytic actions on ss-DNA and RNA, endo-exonuclease has a number of quite different properties. It binds relatively strongly to DEAE-Sepharose and it binds to ss-DNA–cellulose in the presence of EDTA. More importantly, it acts exonucleolytically, rather than endonucleolytically, on ds-DNA (see above). The exonuclease activity has been found to be processive, and both this property and the ss-DNA-binding property have been found to be protease-sensitive (observations of T. Y.-K. Chow and M. J. Fraser in this laboratory). We have observed that when mitochondria are aged in vitro or stored frozen and then extracted, nuclease with the properties described by Linn and Lehmann (17) did indeed appear, but endo-exonuclease was simultaneously lost. The presence of the proteinase inhibitor, PMSF, prevented these changes from taking place. All of these observations would be simply reconciled if the mitochondrial endonuclease described earlier (17) were derived from endo-exonuclease through proteolysis, a possibility currently under investigation. On the other hand, the observations could be explained by a simultaneous proteolytic inactivation of endo-exonuclease and an activation of an unrelated latent endonuclease.

The intracellular localization of the inactive (tryspin-activatable) endo-exonuclease in the cytosol and mitochondrial inner membrane (Table 2) is consistent with the idea that this protein may be a precursor of at least the active endo-exonuclease in mitochondria. Also consistent with this idea is the observation that the inactive protein is present at a higher level in the mitochondria of *uvs-3* than in wild-type mitochondria, whereas the level of active enzyme is correspondingly very low. The presence of precursors of other mitochondrial proteins in the cytosol of *N. crassa* has been described, for example, by Korb and Neupert (13). The substantial association of inactive endo-exonuclease with microsomal membranes (Table 2) probably resulted from nonspecific adsorption of this hydrophobic protein (15) from the cytosol since all of it was accessible to trypsin (see above).

Active endo-exonuclease is present in appreciable amounts in only one of the two wild-type cell compartments containing DNA, namely, the mitochondrion. The low level in *uvs-3* mitochondria (Table 2) may even have resulted from some proteolytic activation of the precursor during handling and extraction. At any rate, this striking deficiency in the mutagen-sensitive mutant can be interpreted to mean that endo-exonuclease probably plays a role at least in the repair of mitochondrial DNA. It is possible that this role could be in a recombinational type of repair since *N. crassa* mitochondrial DNA undergoes recombination in vivo (20). The biochemical

lesion in *uvs-3* could possibly be in a protease which converts the inactive endo-exonuclease to the active enzyme. Another indirect indication of a protease deficiency is the observation that the spontaneous PMSF-sensitive activation of the inactive endo-exonuclease, which was observed previously by Chow and Fraser (5) to occur in sonic extracts of wild-type mycelia, does not occur under the same conditions in sonic extracts of *uvs-3* mycelia. The virtual absence of endo-exonuclease in nuclei (see above) does not necessarily preclude a role for the enzyme in repair of nuclear DNA if some mechanism exists for the transport of the enzyme into nuclei in response to damage to the DNA, but at present, there is no evidence for such a mechanism.

The unusual finding of the present work is that the same enzyme, endo-exonuclease, is present in two completely different types of cell organelles. Unlike the mitochondrial enzyme, most of the vacuolar endo-exonuclease is not associated with the membrane of the organelle (see above). It seems likely that, despite striking similarities in the enzymatic activities of the two endo-exonucleases, differences will be found to exist between the proteins at the molecular level. A physiological role usually attributed to vacuolar hydrolases is in turnover of intracellular macromolecules (23). A role of vacuolar endo-exonuclease in the turnover of RNA and DNA is possible, but the partial deficiency in vacuolar endo-exonuclease observed in the *uvs-3* mutant suggests a possible additional role in secretion. A fraction of the vacuoles may be targeted for secretory processes as occurs in yeasts (24). The secretion of endo-exonuclease activity from *N. crassa* mycelia growing in sorbose-containing medium was found to be deficient in the *uvs-3* mutant relative to the wild type (7).

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