Proteolysis of the Products of Mitochondrial Protein Synthesis in Yeast Mitochondria and Submitochondrial Particles

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Degradation of mitochondrial translation products in *Saccharomyces cerevisiae* mitochondria was studied by selectively labelling these entities *in vivo* in the presence of cycloheximide and following their fate in isolated mitochondria. One-third to one-half of the mitochondrial translation products are shown to be degraded, depending on the culture growth phase, with an approximate half-life of 35 min. This process is shown to be ATP-dependent, enhanced in the presence of puromycin and inhibited by chloramphenicol. Further, the proteolysis is suppressed by detergents and is insensitive to antisera against yeast proteinases A and B when measured in mitochondria or 'inside-out' submitochondrial particles. It is concluded that the breakdown of mitochondrial translation products is most probably due to the action of endogenous proteinase(s) associated with the mitochondrial inner membrane. This proteinase is inhibited by phenylmethanesulphonyl fluoride, leupeptin, antipain and chymostatin.

In recent years the number of studies concerned with assessing the role of endogenous mitochondrial proteinases in mitochondrial biogenesis has markedly increased (Gear *et al.*, 1974; Wheeldon *et al.*, 1974; Rajwade *et al.*, 1975; Subramanian *et al.*, 1975). The first detailed study on mitochondrial proteinases was carried out by Bartley and co-workers (Alberti & Bartley, 1965, 1969; Bailey *et al.*, 1967). Proteins in isolated rat liver mitochondria were shown to be degraded to acid-soluble products by endogenous neutral proteinase(s). In later studies proteinases differing in substrate-specificity and inhibitor-sensitivity were isolated from mitochondria from human bone-marrow reticulocytes (Aoki *et al.*, 1975) and rat liver (Katunuma *et al.*, 1975; Jušić *et al.*, 1976a).

We have previously reported (Bakalkin *et al.*, 1978) the rapid degradation of mitochondrial translation products in *Saccharomyces cerevisiae* cells. However, the identity of the proteinases responsible for this process remained obscure. Although Wheeldon *et al.* (1974) have ascribed the breakdown of mitochondrially synthesized proteins in isolated rat liver mitochondria to an endogenous proteinase, no evidence for the existence of any such enzyme in yeast mitochondria has so far been available.

The present work demonstrates that a considerable portion of the products of mitochondrial protein synthesis is broken down in isolated yeast mitochondria and 'inside-out' submitochondrial particles

prepared therefrom. The proteolysis is inhibited by phenylmethanesulphonyl fluoride, *p*-chloromercuribenzenesulphonate, antipain, chymostatin and leupeptin. It can be suppressed by ionic (sodium dodecyl sulphate) and non-ionic (Triton X-100) detergents. An interesting characteristic of the process is its dependence on ATP.

Materials and Methods

Chemicals

Azocoll, puromycin, carbonyl cyanide *m*-chlorophenylhydrazone, complete Freund's adjuvant and oligomycin were purchased from Calbiochem, Los Angeles, CA, U.S.A. Cycloheximide, phenylmethanesulphonyl fluoride, *p*-chloromercuribenzenesulphonic acid (sodium salt), succinate, haemoglobin and DEAE-Sephadex A-50 were from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-[4,5-³H]Leucine (sp. radioactivity 250 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Chymostatin, antipain, leupeptin and pepstatin were generously given by Professor H. Umezawa, Microbial Chemistry Research Foundation, Tokyo, Japan.

Culture growth and labelling of mitochondrial translation products

The *Saccharomyces cerevisiae* strain used in this work and the growth conditions were described previously (Luzikov *et al.*, 1971). Unless otherwise

indicated, yeast cells were grown up to the end of the exponential phase and the cell suspension (1 litre) was supplemented with 2 μ mol of undiluted [3 H]leucine and cycloheximide (25 mg). After 1 h incubation with labelled leucine the cells were washed five times at 2°C with non-labelled growth medium containing 2 mM-non-radioactive leucine.

Isolation and incubation of mitochondria and sub-mitochondrial particles

Cell homogenates were prepared as described by Tzagoloff (1969) in a medium containing 0.55 M-mannitol, 2 mM-MgSO₄, 2 mM-leucine and 100 mM-Tris adjusted to pH 7.0 with HCl. A mitochondrial fraction was sedimented quantitatively by a 30 min centrifugation of the homogenate at 20000g. The washed mitochondria were suspended (final concn. 3 mg of protein/ml) in a medium containing 50 mM-KCl, 15 mM-MgCl₂, 10 mM-succinate, 0.2 mM-EDTA, 2 mM-ATP, 1 mM-non-labelled leucine and 30 mM-Tris adjusted to pH 7.0 with HCl. During incubation of mitochondria at 35°C 0.5 ml portions of the suspension were collected and the mitochondrial protein was precipitated by addition of 3 ml of 10% (w/v) trichloroacetic acid.

'Inside-out' submitochondrial particles were prepared by the method of Schatz (1968). Mitochondria were sonicated for 3 $\times$ 10 s at 100 W output in an MSE 100 sonifier. Sidedness of submitochondrial particles was determined electron-microscopically as described by Racker & Horstman (1967). Submitochondrial particles were suspended to a concentration of 1.6–1.9 mg of protein/ml and incubated under the same conditions as were used for the mitochondria.

Radioactivity counting

Radioactivity of the trichloroacetic acid-soluble fraction served as the criterion of the content of free amino acids and low-molecular-weight products of protein degradation. The precipitate was washed with 10% (w/v) trichloroacetic acid and dehydrated acetone to remove lipid-bound radioactive leucine (Wheeldon *et al.*, 1974). The protein was reprecipitated from 1 M-NaOH by the method of Hochberg *et al.* (1972), dissolved in 1 ml of 7.4 M-NH₃ by 60 min incubation at 50°C and transferred into glass scintillation vials containing 10 ml of a toluene scintillator solution [4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene/Triton X-100 (2:1, v/v)]. A 1 ml portion of the trichloroacetic acid-soluble fraction was placed into a scintillator of the same composition. The radioactivity (d.p.m.) measured in the spectrometer was used to calculate that in the total volume of the acid-soluble fraction to estimate the balance between the changes in the radioactivity of the protein and the acid-soluble fractions. The counting of radioactivity was performed in a Nuclear-Chicago mark

III liquid-scintillation spectrometer. The mean efficiencies of counting of 3 H radioactivity in the trichloroacetic acid-soluble fraction and the protein fraction were 30% and 40% respectively. The computerized system was programmed according to the recommended procedure [Mark III Liquid Scintillation System: Model 6880 (1974) Searle Analytic Publication no. 710-717760] to attain the highest counting efficiency and a relative uncertainty of less than 2% at 2 σ setting.

Isolation of yeast proteinases A and B

The yeast was disintegrated in a X-press (Biotec) under a pressure of 20 MPa (2000 kg/cm²) at -20°C. The homogenate was activated at room temperature for 48 h as described by Saheki & Holzer (1975). Proteinases A and B were isolated as described by Betz *et al.* (1974) except that the eluent was supplemented with glycerol to a final concentration of 30% (v/v). Proteinases were stable under the assay conditions at 37°C for at least 1 h. Proteinase A activity was assayed with acid-denatured haemoglobin as a substrate, and proteinase B activity was assayed with azocoll as a substrate by the method of Lenney (1975).

Preparation of antisera

Proteinases A and B were suspended in 0.9% NaCl at a concentration of 2 mg/ml, and then mixed with complete Freund's adjuvant (1:1, v/v). Antigen emulsions (2 ml) were injected into rabbit groin lymphatic glands; 1 week later another 7 ml of the same emulsion was injected. Then about 6 weeks after the first injection 100–120 ml of blood was withdrawn from the rabbit heart and serum was prepared. The control sera were obtained from the blood of the same rabbits before the first immunization; γ -globulin fraction was prepared as described by Somlo (1962).

Measurement of protein content

Protein content was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, or spectrophotometrically, by measuring the difference in absorption at 280 and 260 nm (Warburg & Christian, 1942).

Reproducibility

The data presented are the averages for four independent experiments. The overall variance was within $\pm 5\%$ of the mean.

Results

Degradation of the products of mitochondrial protein synthesis in isolated mitochondria

The breakdown of mitochondrial translation products (i.e. polypeptides synthesized on mitochondrial ribosomes *in vivo* in the presence of cycloheximide) in isolated yeast mitochondria was

Fig. 1. Changes in the radioactivity of the products of mitochondrial protein synthesis (a) and trichloroacetic acid-soluble components (b) during incubation of isolated yeast mitochondria

Mitochondrial translation products were labelled at the exponential (○) or early stationary (●) phase of culture growth by a 1 h incubation of the cell suspension with [^3H]leucine (0.5 mCi/litre) and cycloheximide (25 mg/litre). Then mitochondria were isolated and incubated at 35°C in a medium containing 2 mM-ATP, 15 mM-MgCl₂ and 10 mM-succinate. At indicated times samples of mitochondrial suspension were withdrawn and trichloroacetic acid was added to a final concentration of 10%. Acid-soluble and acid-insoluble fractions were treated as described in the Materials and Methods section and their radioactivities were counted.

studied by monitoring the decrease in the content of the label in the mitochondrial protein and its accumulation in the acid-soluble fraction during incubation of mitochondria in the presence of ATP, Mg²⁺ and succinate. Fig. 1(a) shows that the radioactivity of the polypeptides decreased by 50% or 30% (in mitochondria isolated from cells labelled and harvested in the exponential and early stationary phases respectively) during 70 min of incubation and then remained practically constant for the subsequent 50 min. Concomitantly the label was accumulated in the acid-soluble fraction (Fig. 1b). The degradation rate was maximal at pH 7.0–7.5. These findings are indicative of proteolysis of mitochondrial translation products. The quantitative correlation between the changes in the radioactivities of mitochondrial protein and acid-soluble products was observed in all experiments. Therefore only the radioactivities of

mitochondrial protein are presented for the subsequent experiments described below.

Can vacuolar proteinases be responsible for the degradation in vitro of mitochondrial translation products?

The question arises whether the observed breakdown of mitochondrial translation products could be due to contaminating cytoplasmic proteinases. Vesicles containing proteinases capable of rapidly hydrolysing mitochondrial translation products were detected by Michel *et al.* (1976) in mitochondrial preparations from *Neurospora crassa*. These proteinases were activated by Triton X-100. In the present work, however, 0.1% and 0.5% Triton X-100 in the incubation medium suppressed the proteolysis of mitochondrial translation products by 80% and

Fig. 2. Effect of detergents on the proteolysis of mitochondrial translation products

Mitochondria were incubated as described in the legend to Fig. 1. (a) ●, Control conditions; ○, plus 0.1% sodium dodecyl sulphate; ▲, plus 1.0% sodium dodecyl sulphate. (b) ●, Control conditions; ○, plus 0.1% Triton X-100; ▲, plus 0.5% Triton X-100.

100% respectively. Sodium dodecyl sulphate was ineffective at 0.1% and caused 80% inhibition at 1% (Fig. 2). These data strongly suggest that the proteolysis of mitochondrial translation products did not result from the action of proteinases contained in vacuoles that might contaminate the mitochondrial fraction.

More direct evidence against the participation of cytoplasmic proteinases in the degradation of mitochondrial translation products was obtained in experiments with antisera against proteinases A and B.

Saccharomyces cerevisiae cells are known to possess endopeptidase A (EC 3.4.23.8) and endopeptidase B (EC 3.4.22.9), which are localized in vacuoles (Wiemken & Matile, 1967; Saheki & Holzer, 1975) and considered to be marker enzymes of these structures. In contrast with vacuolar carboxypeptidase Y, they have been shown to hydrolyse some yeast enzymes (Jušić *et al.*, 1976b). The specific activities of proteinases B and A in the mitochondrial preparation used in the present work were less than 1% and 0.5% respectively of those in the cell homogenate.

The possibility remained, however, that very small amounts of vacuolar proteinases might contaminate the mitochondrial fraction and be able to induce proteolysis of the mitochondrial translation products. If this were the case, specific antibodies against proteinases A and B would inhibit the proteolysis.

To test this suggestion, specific antisera were prepared against isolated proteinases A and B. Anti-(proteinase A) serum and anti-(proteinase B) serum did not react with proteinases B and A respectively, i.e. there were no cross-reactions. Complete inhibition of proteinase activities was attained at antigen/antiserum protein ratios of 1:8 for proteinase A and 1:7 for proteinase B.

Mitochondria were preincubated with the antisera for 30 min at room temperature in 0.05 M-Tris adjusted to pH 7.0 with HCl and then placed into the assay medium containing ATP, Mg^{2+} and succinate. The concentrations of antisera were sufficient to completely inhibit proteinases A and B in amounts equivalent to up to 30% of mitochondrial protein; however, the proteolysis of mitochondrial translation products was not suppressed by anti-(proteinase A) serum or by anti-(proteinase B) serum (Fig. 3a).

Proteolysis of mitochondrial translation products in submitochondrial particles

As indicated by Fig. 3(b), during incubation of submitochondrial particles in a medium containing ATP, Mg^{2+} , succinate and non-labelled leucine about 20% of mitochondrial translation products labelled in the presence of cycloheximide are subject to proteolysis with an approximate half-life of 35 min, similar to that observed with mitochondria. The rate

Fig. 3. Changes in the radioactivity of mitochondrial translation products during incubation of mitochondria and submitochondrial particles: effects of anti-(proteinase A) and anti-(proteinase B) sera

Preparations were incubated as described in the legend to Fig. 1. (a) ●, Mitochondria (2mg/ml); ○, plus anti-(proteinase A) serum (7.2 mg/ml); ▲, plus anti-(proteinase B) serum (6.3 mg/ml). (b) ●, Submitochondrial particles (1.6mg/ml); ○, plus anti-(proteinase A) serum (3.9 mg/ml); ▲, plus anti-(proteinase B) serum (3.4 mg/ml).

of the breakdown of mitochondrial translation products in submitochondrial particles was not affected by the addition of the supernatant remaining from the preparation of the submitochondrial particles. Further, this process was insensitive to anti-(proteinase A) and anti-(proteinase B) sera (Fig. 3b), as with mitochondria.

These results show that yeast mitochondria possess an endogenous proteinase associated with the inner membrane. No explicit interpretation can at present be given to the lower efficiency of the degradation of mitochondrial translation products in submitochondrial particles as compared with mitochondria; it might reflect some disturbances of the very complex enzyme-substrate interactions in the membrane during the preparation of submitochondrial particles.

Effects of various proteolytic inhibitors on the breakdown of mitochondrial translation products

The data presented in the preceding sections suggest the participation of a mitochondrial proteinase in the degradation of mitochondrial translation products. The type of proteinase involved may be

Table 1. *Effect of synthetic and natural inhibitors on the activities of proteinases A and B and proteolysis in mitochondria and submitochondrial particles*

The assays of proteinases A and B were performed with haemoglobin and azocoll as substrates respectively. Proteolysis in mitochondria and submitochondrial particles was recorded as described in the Materials and Methods section.

Inhibitor	Concn.	Inhibition (%)			
		Proteinase A	Proteinase B	Mitochondrial proteolysis	Proteolysis in submitochondrial particles
Phenylmethanesulphonyl fluoride	0.2mM	0	100	76	—
	1.0mM	0	100	85	—
<i>p</i> -Chloromercuribenzenesulphonate	1.0mM	0	100	75	—
	2.0mM	0	100	89	—
Pepstatin	1.0 µg/ml	100	0	0	0
Chymostatin	2.0 µg/ml	0	100	83	—
Antipain	2.0 µg/ml	0	100	95	—
Leupeptin	2.0 µg/ml	5	0	55	60
Chloramphenicol	4.0mg/ml	0	0	95	—

Fig. 4. *Effects of puromycin and chloramphenicol on proteolysis of mitochondrial translation products*
For incubation conditions see the legend to Fig. 1.
●, Control; ○, plus puromycin (10 µg/ml); △, plus chloramphenicol (4 mg/ml).

revealed by analysing the inhibitor-sensitivity of this process. Table 1 shows that phenylmethanesulphonyl fluoride, an inhibitor of serine proteinases, in concentrations of 0.2mM and 1mM inhibited proteolysis by 76 and 85% respectively, and *p*-chloromercuribenzenesulphonate, a thiol-blocking reagent, in concentrations of 1.0mM and 2.0mM inhibited the process by 75 and 89% respectively. Pepstatin, a microbial oligopeptide inhibitor of acid proteinases (Umezawa, 1973), does not affect intramitochondrial proteolysis (Table 1). The inhibitor suppresses proteinase A and is completely ineffective with proteinase B, which agrees with the data of Saheki & Holzer (1975). Pronounced suppression of the proteolysis of mitochondrial translation products was observed

in the presence of chymostatin and antipain, which are potent inhibitors of isolated proteinase B (see Table 1). High sensitivity of proteinase B to the above inhibitors was reported by Ulane & Cabib (1976).

Of particular interest is the fact that intramitochondrial proteolysis was significantly inhibited by leupeptin, which did not affect proteinases A and B (Table 1). Finally, proteinases A and B are insensitive to chloramphenicol, in the presence of which proteolysis of the products of mitochondrial translation could hardly be observed.

The incomplete inhibition of the breakdown of mitochondrial translation products in mitochondria by leupeptin and the ineffectiveness of pepstatin might result from the limited permeability of mitochondrial membranes to these compounds. However, the results of experiments with submitochondrial particles also demonstrate (Table 1) the lack of inhibitory action of pepstatin and 60% inhibition by leupeptin.

Effect of puromycin and chloramphenicol on degradation of the products of mitochondrial protein synthesis

According to Wheeldon *et al.* (1974), nascent polypeptides released from mitochondrial ribosomes are particularly sensitive to proteolysis on incubation of rat liver mitochondria labelled *in vitro*. As shown in Fig. 4, the rate and extent of degradation of proteins labelled *in vivo* increase after the incubation medium is supplemented with puromycin, which is known to induce the release of non-terminated polypeptides from mitochondrial ribosomes. Since polypeptide synthesis from endogenous amino acids took place in the present experimental conditions (results not shown), the above results support the suggestion made by Wheeldon *et al.* (1974).

In the presence of chloramphenicol degradation of mitochondrial translation products was markedly

Fig. 5. Dependence of proteolysis of mitochondrial translation products on ATP

●, Control; ○, without ATP, succinate and Mg²⁺; ■, plus oligomycin (2 µg/ml); △, plus carbonyl cyanide *m*-chlorophenylhydrazone (1 µM). Incubation conditions were as indicated in the legend to Fig. 1.

suppressed (Fig. 4). Though this effect is hard to interpret, a possible explanation, which can be inferred from the data of Grisolia *et al.* (1977), may be a direct inhibition of the mitochondrial proteinase by chloramphenicol.

Dependence of proteolysis of mitochondrial translation products on ATP

One of the peculiar features of the proteolysis of mitochondrial translation products is its dependence on ATP. As Fig. 5 shows, during incubation of yeast mitochondria in an ATP-deficient medium, decrease in radioactivity was less than 10% of that in the control. This effect was not studied in detail in the present work. It should only be noted that the effect of ATP was not associated with energy transformation in mitochondria, since neither carbonyl cyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation, nor oligomycin, an inhibitor of the adenosine triphosphatase complex, affected proteolysis of the mitochondrial translation products in the presence of ATP, succinate and Mg²⁺.

According to the data presented in Figs. 1 and 5, degradation of polypeptides synthesized on mitochondrial ribosomes slowed down after 30 min incubation of mitochondria. It could be assumed that this was due to exhaustion of ATP in the incubation medium. Further experiments showed, however, that repeated addition of ATP did not influence this process.

Discussion

In recent years researchers have shown increasing interest in the mitochondrial proteinases, which may be responsible for the catabolism of mitochondrial proteins and perform regulatory functions in mitochondrial formation (see, e.g., Gear *et al.*, 1974; Rajwade *et al.*, 1975; Subramanian *et al.*, 1975). The variety and functional scope of mitochondrial proteinases are still far from being clear. In particular, according to Katunuma *et al.* (1975), rat liver mitochondria possess a proteinase breaking down apoprotein moieties of pyridoxal-dependent enzymes. Jušić *et al.* (1976a) reported a liver mitochondrial proteinase hydrolysing histones and chromosomal non-histone proteins. Haas *et al.* (1977) demonstrated that submitochondrial particles obtained from liver mitochondria catalyse the hydrolysis of a proteinase synthetic substrate, acetyltyrosine ethyl ester. Rat liver mitochondria were shown by Hare (1978) to contain a membrane-bound proteinase digesting insulin.

Especially interesting in the light of the problem of mitochondrial biogenesis would be a proteinase responsible for the proteolysis of mitochondrial translation products. The latter are believed by several workers (Tzagoloff, 1971; Ebner *et al.*, 1973; Bertrand & Werner, 1977) to play an important role in the assembly of catalytic complexes of the mitochondrial inner membrane. Wheeldon *et al.* (1974) were the first to demonstrate that nascent polypeptides in isolated rat liver mitochondria are subject to proteolysis with a half-life of about 20 min.

The main purpose of the present work was to elucidate whether yeast mitochondria possess a proteinase responsible for the breakdown of mitochondrial translation products. The Results section presents arguments in favour of the existence of such a proteinase. Firstly, it is shown that a proportion of polypeptides synthesized on mitochondrial ribosomes undergoes proteolysis in isolated yeast mitochondria (Fig. 1). The mitochondrial fraction seems to be devoid of vacuolar enzymes, as judged by the assays of proteinases A and B, since the latter can be considered marker enzymes of the vacuoles and, according to Holzer *et al.* (1975), all known yeast cytoplasmic proteinases are localized in vacuoles. Other indirect evidence against the participation of cytoplasmic proteinases in the digestion of mitochondrial translation products in isolated yeast mitochondria is the lack of any stimulating effect by detergents (sodium dodecyl sulphate and Triton X-100; Fig. 2). Indeed, activation of proteolysis by detergents should have been observed if the mitochondrial fraction were contaminated with vacuoles, according to Michel *et al.* (1976). It could still be thought that degradation was caused by

minor amounts of vacuolar proteinases undetectable in standard assays. However, the insensitivity of the proteolysis to anti-(proteinase A) and anti-(proteinase B) sera (Fig. 3a) signifies that the corresponding proteinases play no role in the breakdown of polypeptides synthesized on mitochondrial ribosomes. Finally, the very fact of the antisera-insensitive proteolysis in submitochondrial particles (Fig. 3b) strongly suggests that mitochondria possess their own proteinase associated with the inner membrane. With this in view, it is easily understood why Triton X-100 is inhibitory, since labilization of the membrane may result in removal of the proteinase or hinder the enzyme-substrate interaction.

The results obtained demonstrate that the mitochondrial proteinase is suppressed by several proteolytic inhibitors, namely phenylmethanesulphonyl fluoride, leupeptin, antipain and chymostatin, but insensitive to pepstatin (Table 1). The comparison of these results with the inhibitor-sensitivities of vacuolar endopeptidases A and B and carboxypeptidase Y leads one to the conclusion that none of these enzymes participates in the breakdown of mitochondrial translation products. Indeed, proteinase A is not affected by the inhibitors of serine proteases (Saheki & Holzer, 1975), proteinase B is not inhibited by leupeptin (Lenney, 1975) and carboxypeptidase Y is insensitive to any of the above peptide inhibitors (Lenney, 1975). It should be noted that, according to Umezawa (1977), these naturally occurring inhibitors are ineffective towards aminopeptidases and carboxypeptidases from various sources.

The proteolysis of mitochondrial translation products in yeast mitochondria shares the following features with that described by Wheeldon *et al.* (1974): (i) it is enhanced in the presence of puromycin, (ii) it is dramatically inhibited by chloramphenicol and (iii) it is ATP-dependent (Figs. 4 and 5).

Thus the present work demonstrates a proteinase inherent in yeast mitochondria and associated with the mitochondrial inner membrane. The enzyme efficiently digests mitochondrial translation products and, since the latter are believed to play a key role in the assembly of the catalytic complexes of the mitochondrial inner membrane (Tzagoloff, 1971; Ebner *et al.*, 1973; Bertrand & Werner, 1977), may perform regulatory functions in mitochondrial formation.

Rapid degradation of mitochondrial translation products *in vivo* has been described in a previous paper from this laboratory (Bakalkin *et al.*, 1978). The data now reported may benefit the assessment of the role of the endogenous mitochondrial proteinase in this process.

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